# RELEASE AND ACTIONS OF NEUROTRANSMITTER MOLECULES AT NEUROGLANDULAR JUNCTIONS IN COCKROACH SALIVARY GLANDS.

by

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THESIS

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Dedicated to the memory of

James Tindal of Glasgow,

graduate of this College 1834.

CONTENTS

## SUMMARY

The innervation of the salivary gland of the cockroach Nauphoeta cinerea (Olivier) has been investigated with the use of light and scanning electron microscopy (SEM). Light microscopy revealed the presence of a dual innervation arising from the ventral nerve cord and the stomadeal nervous system; the principal innervation is that from the ventral nerve cord which passes to the gland via the reservoir ducts. Branches of these nerves form a plexus on the acinar surface, the axons of which exhibit swellings at irregular intervals. The presence of this plexus and the axonal swellings was confirmed by SEM both in normal glands and in those in which the basement membrane had been removed by means of an HCl-collagenase digestion method. Cell bodies associated with the larger axons of the duct nerves were identified in the sub-oesophageal ganglion using an axonal filling method employing cobalt chloride and horseradish peroxidase. No acinar plexus was apparently formed by branches of the stomatogastric nerve associated with the gland. Other branches of this nerve were connected with a network of multipolar neurones on the surface of both salivary reservoirs.

Intracellular recordings from the gland cells revealed that a hyperpolarizing response evoked by electrical stimulation of the duct nerve was graded according to the number of stimuli. The biogenic amines, adrenaline, dopamine, noradrenaline, 5-hydroxytryptamine and octopamine,

produced dose-dependent hyperpolarizing responses. A quantitative study of the inhibition by phentolamine on the response to nerve stimulation and the bath applied agonists was made. The investigation showed that phentolamine discriminates between two kinds of receptor in this gland, one binding 5-hydroxytryptamine and the other combining with the catecholamines and the neurotransmitter. The inhibition appeared to be competitive and measures of phentolamine's affinity constant gave values of 0·015 $(\mu M)^{-1}$ and 1 $(\mu M)^{-1}$ for each type of receptor respectively. It was concluded that the neurotransmitter in the cockroach salivary gland was probably dopamine.

# GENERAL INTRODUCTION

The foundations for the study of modern insect anatomy and physiology were laid down by Malpighi (1628 - 1694) and embodied in his treatise on the silkworm,"De Bombyce" (published in London in 1699 under the auspices of the Royal Society). This remarkable and entirely original account marked the start of a new era in the study of invertebrate biology. Jan Swammerdam (1637 - 1680) built on and added to the knowledge gained by Malpighi and incorporated much of it in his own great posthumous work "Biblia Naturae" (assembled by Boerhaave and published in Leyden in 1737). It is in this volume that the first reliable accounts of the salivary glands of insects can be found. He accurately described both the tubular glands of the gadfly, tracing their development from larva to adult, and the acinar glands of the cheese mite (Piophilia casei, L.). He appeared a little perplexed on the nature of the acini describing them as "a fatty matter but of a peculiar nature", but has little doubt as to their function suggesting that they "may serve to moisten the food of this worm (mite) and thereby render it of easy digestion".

The most outstanding study of insect anatomy of the 18th century was that of Pierre Lyonet (1707 - 1789), who published in 1762 his famous monograph entitled "Traité anatomique de la Chenille qui ronge le Bois de Saule". In this elegant study on the anatomy of the goat moth larva (Cossus cossus, L.) Lyonet gave a detailed account of the salivary apparatus comprising paired silk glands and

associated accessory organs, and described for the first time the source and distribution of their innervation, the two distinct cellular layers of the reservoirs and what appears to be the first experimental attempt to investigate the digestive function of saliva in insects. His observations are all the more remarkable when one considers he used only a simple lens mounted in a wooden frame.

At the start of the 19th century studies in insect anatomy and physiology received a new impetus consisting mainly of the search for correspondences between insects and mammals. This can be attributed to the combined influences of Linnaeus (Systema Naturae, 1735 - 58) and Fabricius (Systema Entomologiae, 1775) on taxonomy and to reborn interest in comparative anatomical studies as typified by Cuviers "Lectures in Comparative Anatomy" (1799). These influences can be seen in the works of Kirby and Spence (1826), Staus -Durkheim (1828), an associate of Cuvier, Burmeister (1832) and Dufour (1835). These authors all gave general accounts of salivary glands and their role in the digestive processes of insects, and noted that the saliva is generally a clear watery fluid, usually alkaline (adduced by its action on tumeric paper) and that it has some kind of chemical action on food, Burmeister (1832) comparing its action to that of boiling vegetables.

It is however Dufour (1835) who should be credited with the first description of the salivary gland of a

cockroach (Blatta orientalis, L.). He distinguished it as two integral parts, lying on the ventral surface of the crop and composed of the glandular or acinar portion and the reservoirs. Although he observed that the acini were arranged in several groups, the ducts from which on each side united into a common secretory duct which emptied into the mouth beneath the tongue, he failed to note the relationship between the reservoir ducts and the secretory ducts.

That much of this early work is of a general and limited nature is due largely to the inadequacies of the methods available at the time. With the introduction of the achromatic compound microscope in about 1840 and the application of more rigorous biochemical techniques Basch in 1858 was able to extend the work of Dufour on the cockroach salivary gland.

Basch accurately described the general structure of the gland and established the exact relationship between the efferent ducts and the reservoirs but curiously assumed that the main salivary duct terminated in the pharynx. He observed that the acini were composed of three layers, an external structureless envelope, an intimate tunic or membrane propria, which also accompanied the secretory ducts, and a middle layer composed of granular nucleated cells. These cells often formed fine secretory ducts or "spiechelsackes" which opened into the secretory duct. The ducts were seen to comprise a single layer of epithelial cells and an internal chitinous membrane formed of spiral

filaments like those of tracheae.

With regard to the biochemistry of saliva Basch found that it was able to convert starch into sugar (at room temperature), gave an acid reaction and on addition of a weak solution of HCl had the ability to digest blood fibrin. Although Basch was found to be incorrect on the two latter points (Plateau, 1876; Griffiths, 1885; Wigglesworth, 1927 a,b) his experiments deserve notice as the first attempts to investigate the digestive fluid in insects using quantitative biochemical methods. The biochemistry of digestion in insects was worked out in the classic papers of Plateau (1874 - 76) who reproduced on a small scale all the contemporary work on vertebrate digestion and established the presence of the main types of digestive enzymes. Plateau concluded that the saliva of the cockroach changes starch into glucose, but the saliva is not acid, it is either neutral (B. orientalis) or alkaline (Periplaneta americana, L.) and that any acidity found in the crop is due mainly to the ingestion of acidic food.

Following the improvement in microscope design came the systematic development of histological techniques which enabled Kupffer (1874) to give an accurate description of the fine structure of the salivary glands of the cockroach B. orientalis. He correctly observed that the acini were composed of two cell types which he named "peripheren zellen" (peripheral cells) and "centralen zellen" (central cells). The peripheral cells were distinguished from the central cells by being situated directly beneath the

basement membrane, usually in pairs; each cell containing a retort-shaped capsule joined by a chitinous tubule to the efferent duct. In addition Kupffer gave the first detailed account of the innervation noting that the glands were innervated from two sources, the ventral nerve cord and the stomatogastric nervous system. However he incorrectly assumed that branches arising from the stomatogastric nerve exclusively innervated the acini and that those arising from the ventral nerve cord were concerned solely with the control of the reservoirs, which he likened to a chyliferous stomach. On the general arrangement of the ducts he repeated Basch's error in thinking that they emptied directly into the pharynx.

Huxley (1877) in his text book on the invertebrata corrected this point and established that the main salivary duct opened beneath the lingua into the hypopharynx. Cholodkowsky (1881) confirmed this arrangement in the cockroaches B. orientalis, B. germanica (L.) and P. americana. In addition he showed that the innervation from the ventral nerve cord arose via a small pair of nerves on the posterior margin of the sub-oesophageal ganglion and passed down the reservoir ducts to supply not only the reservoirs as thought by Kupffer but the glandular portion as well; thus establishing that the acini received an innervation from both the ventral nerve cord and the stomadeal nervous system.

Hofer (1887) using sectioned material confirmed and extended the previous observations of Kupffer on the fine

structure and Cholodkowsky on the source of the innervation. On the general structure of the gland Hofer noted that each reservoir was composed of two layers, an outer epithelium and a chitinous lining and that the mouth of the reservoir was controlled by a set of muscles. In an extensive study of the innervation of the gland, which incidentally included a detailed description of the anatomy of the brain, he traced the course of the salivary duct nerve within the sub-oesophageal ganglion, (following a similar course as that found in N. cinerea, see later) and attempted to trace the origin of the salivary nerves emanating from the stomadeal nervous system. By comparing the histological changes in the structure of acinar cells obtained from normal and starved cockroaches Hofer was able to conclude that both the peripheral and central cells contributed in some way to the secretion formed by the salivary glands. However he erroneously assumed that the peripheral cells transmitted the central cell secretion into the efferent duct.

Lebedeff (1899), in a comprehensive account of the microphysiology of the salivary glands of B. orientalis, clearly demonstrated the independent activity of the acinar cells, observing that mucin was exclusively produced by the central cells and separately secreted into the terminal portion of the efferent duct. This he achieved by employing advanced histochemical methods in conjunction with intra-abdominal injection of pilocarpine to intensify glandular activity. In addition he showed that the

peripheral cell capsule was lined with a brush border, suggesting that such an arrangement would facilitate the passage of the cellular secretion into the capsule cavity. With remarkable insight he also suggested that not only the peripheral cells but also the efferent duct cells lying close to the acini participated in the activity of the glands by producing a dilute and watery secretion.

Thus at the end of the 19th century much of the fine structure of the cockroach salivary gland had been elucidated at the level of the light microscope leaving little to be added until the advent of transmission electron microscopy some fifty years later. However views on the nervous control of salivary secretion reflected much of the controversy on the structure and function of the nervous systems generated by vertebrate physiologists of the period. The principal theories on the structure of nervous systems were, the reticular theory propounded by Gerlach (1871) and Golgi (1885) which assumed a continuity of structure between the nerve cells via a syncytial arrangement of their dendritic processes, and the specific neurone theory formulated by Waldeyer (1891) based on the work of Ramon Y Cajal in the eighties and nineties, (using a modification of Golgi's silver stain) which demonstrated the independence of neurocellular structure (see Schäfer, 1900 for contemporary review and references).

The neurone theory hinged on the principal assumption that nerve cells entered into functional connection with each other (or their effector organs) by contiguity not

continuity. Sherrington (1897) recognised this special connection between nerve cells and suggested the term synapse to describe it. A little later he realised the functional implications of such a structure to explain the unidirectional nature of mammalian spinal reflexes (Sherrington, 1900), thereby giving physiological significance to the neurone theory. The controversy was finally resolved in favour of the specific neurone theory (see review by Bodian, 1942) and gained an unassailable position with the pioneering work of Palade and Palay (1954) using the electron microscope. As a footnote to the controversy concerning the neurone theory it is of interest to note that as early as 1878 Schäfer, using an invertebrate preparation (the sub-umbrellar plexus of Aurelia aurita, L.) provided clear evidence of a discontinuity not only between the individual fibres of the plexus but also between the fibres and the underlying muscle, suggesting that expansions observed on the axons represented "a primitive form of motorial end plate".

The dispute between the structural concepts was parallelled by two opposing theories on the functional aspects of nervous systems. Since it was well established, following the famous 18th century debate between Galvani and Volta, that electrical events were involved in nerve transmission, the opposing theories centred on the speculations on the nature of transmission across junctional regions. Du Bois-Reymond (1877), who discovered the action potential of nerve, was the first to suggest that

junctional transmission could either be electrical or chemical. Thus the reticular concept of neuronal structure could be allied with the electrical theory of synaptic transmission, since the transmission of electrical activity between cells is essentially a continuation of the process by which this activity is conducted within the boundaries of individual nerve fibres. The chemical theory of synaptic transmission could be invoked to explain the mediation between the discontinuous structures proposed by the specific neurone concept.

Both Kupffer (1874) and Hofer (1887) interpreted their observations on the terminal innervation of the cockroach salivary gland in the light of the reticular theory. Kupffer stated that the acinar nerves, on passing under the basement membrane, branched profusely and dispersed into a network of fine neurofibrils forming a plexus within the peripheral cells. Hofer, whilst in general agreement with Kupffer on the location of the nerve terminals, denied the existence of the intracellular plexus and maintained that the neurofibrils merged imperceptibly with the protoplasm of the peripheral cells. Whilst neither of these authors provided clear evidence on the mode of action of these nerves, their recognition of the peripheral cells as the principle sites of secretion led them to believe that the specific areas of contact of the acinar axons were restricted to these cells, and by analogy with the suggestion of Pflüger who produced similar evidence for the vertebrate salivary gland, Hofer

firmly believed in direct electrical connection between them.

Of the early workers on the cockroach salivary gland only Lebedeff (1899) gave an account consistent with the alternative view on nerve terminal structure, stating "With every desire to trace the penetration of these nerves into peripheral cells, I have always noticed between them and the latter a very distinct boundary, consisting of the same membrane propria." Lebedeff, on the authority of Heidenheim; also rejected Pflüger's evidence on the basis of the failure of other workers to confirm his findings in the vertebrate salivary gland.

In addition Langley (1878), working in Heidenheim's laboratory, whilst studying the effects of the secretogogue pilocarpine and its antagonism by atropine on the sub-maxillary gland of the cat provided evidence for the chemical nature of synaptic transmission in the vertebrate salivary gland, assuming the presence of "some substance or substances in the nerve endings or gland cell with which atropine or pilocarpine are capable of forming compounds". A little later (1880) he was to be more definite on the site of action stating that the gland cells consisted of different parts, one stimulated by physostigmine and antagonised by atropine and another stimulated by the action of the sympathetic innervation.

It is clear from Lebedeff's (1899) paper that he was fully conversant with the current views on vertebrate salivary secretion and although he makes no allusions to

the action of the acinar nerves his use of pilocarpine to stimulate secretory activity could perhaps be interpreted as a tacit assumption in a belief in the chemical nature of synaptic transmission in the cockroach salivary gland.

Nevertheless the case for chemical transmission at any junction was far from proven although the later work of Langley (1905 - 1909) on the skeletal neuromuscular junction provided a certain measure of support. Langley showed that 'receptive substances' for nicotine and curare were localised beneath the nerve terminals. Furthermore, following the suggestion by Elliot (1904) that sympathetic nerve impulses released minute amounts of adrenaline-like substance at the junctional regions of smooth muscle, Langley felt that the nerves at the skeletal neuromuscular junction affected these 'receptive substances' by the release of a chemical mediator. In 1907 Dixon, noting the similarity between the effects of the alkaloid muscarine and stimulation of the vagus, proposed the idea that the vagus liberated a muscarinic compound which acted as a chemical transmitter of its impulses, concluding that 'excitation of a nerve induces the local liberation of a hormone which causes specific activity by combination with some constituent of the end organ, muscle or gland'.

However, it was the experiments of Loewi, begun in 1921 (summarised by Loewi, 1933), that provided clear evidence for the existence of chemically mediated synaptic activity. In the now classic experiments using two isolated frog hearts, in which the perfusion fluid flowed

from one to the other, Loewi showed that stimulation of the vagus of one heart led to either a decrease or increase in the heart rate of both organs. Since the second heart was only connected by perfusion fluid these effects must have been caused by the presence of certain substances released from the stimulated nerves which Loewi termed 'vagus stoff' and 'accelerans stoff' respectively.

Evidence that acetylcholine (ACh) was the parasympathetic mediator of 'vagus stoff' was subsequently presented by Chang and Gaddum (1933) and Gaddum (1936). Dale and Dudley (1929) had previously shown that ACh was a naturally occurring substance. In the same period Dale and Feldburg (1934) and Dale, Feldburg and Vogt (1936) also demonstrated that ACh was likely to be the transmitter at the neuromuscular junction.

The upshot of the long series of experiments by Dale and his colleagues was the formulation by later workers (e.g. Paton, 1958) of a set of criteria for the demonstration of a chemically operating synapse. These can be summarised as follows:- (a) that the presynaptic neurone can synthesize the transmitter and (b) release it in a pharmacologically identifiable form in response to nervous stimulation; and that its action on the postsynaptic cell should (c) reproduce the specific events of normal transmission and (d) that there is an identity of pharmacology between the presumed transmitter and synaptic activity. In addition there should exist an inactivating or removal mechanism for the transmitter. (Curtailment of the local

action of ACh by the enzyme acetylcholinesterase (AChe) was recognised by Dale and his colleagues but the first reliable demonstration of its presence at nerve terminals, using a specific histological technique, was not provided until the work of Koelle and Friendenwald in 1949).

Meanwhile the identity of the accelerans stoff proved more difficult to establish. In the same year as Loewi's discovery Cannon and Uridil (1921) reported that the liver, upon stimulation of the hepatic nerves, released an adrenaline-like substance which increased the heart rate and blood pressure but failed to dilate the pupil of the eye, a property of adrenaline established by Langley (1901). Since it could not be readily identified with adrenaline Cannon and Bacq (1931) proposed the term sympathin and to account for its anomalous effects, Cannon and Rosenblueth (1933) later postulated that the transmitter released from sympathetic nerves was adrenaline, which combined with some constituent of the effector cell to form inhibitory (I) or excitatory sympathin (E) or both. The earlier suggestions of Barger and Dale (1910), subsequently supported by the work of Bacq (1934), and Greer, Pinkston, Baxter & Brannon (1938), that noradrenaline conformed better with the actions of the sympathetic transmitter were widely disregarded, until it was shown by Von Euler (1946) that adrenergic nerves contained not adrenaline (AD) but noradrenaline (NA). Release of NA following stimulation of sympathetic nerves was demonstrated by Peart (1949). (The terms adrenergic and

cholinergic were coined by Dale (1934) to describe the functional differences between sympathetic and parasympathetic nerves respectively).

Thus the 'curiously anomalous' effect on the iris became readily explained by the recognition that NA was the mediator of adrenergic nerve action and the two broad classes of adrenergic effects could be accounted for by two principal receptors, $\alpha$ and $\beta$, proposed by Ahlquist (1948), each having a different sensitivity to various agonists and antagonists.

Another approach to understanding the nature of the sympathetic mediator had its origins in the biochemical studies on the synthesis of adrenaline in the mammalian body (see review by Blaschko, 1957). The outcome of this work was to show that not only did NA serve a dual role, as both precursor to AD and chemical mediator in sympathetic nerves, but that its immediate precursor in the biosynthetic pathway, dopamine, was itself suggested as a possible transmitter candidate.

Termination of the local action of the adrenergic transmitters, by analogy with the actions of ACh at the neuromuscular junction, was thought to be controlled by the enzyme mono-amine oxidase, the actions of which were characterised by Blaschko, Richter & Schlossmann (1937), and in the early 1950's much fruitless effort was expended to establish that this was the case (see Blaschko, 1952), until the existence of neuronal uptake as a mechanism for the synaptic inactivation of the catecholamines was

established by Axelrod and his co-workers in the early sixties (see review by Iversen, 1967).

A similar extension of ideas from the cholinergic transmitter theory led early workers to consider the possible role of ACh as a central transmitter (see Dale, 1935); nevertheless most physiologists were reluctant to believe in central chemical transmission on the assumption that such a mechanism could not explain the rapid transfer of signals in the vertebrate CNS (see Eccles, 1946, 1948).

With regard to the early work to establish the nature of synaptic transmission in the invertebrates the common earthworm (Lumbricus terrestris) and the medicinal leech (Hirudo medicinalis) occupy a unique position. Gaskell (1914, 1919) suggested that the chromaffin cells of these annelids released an adrenaline like compound which acted as regulator of the pulsations of the lateral blood sinuses. For many years this was the accepted view (see for example Ziller-Perez, 1942), until the more recent specific histochemical methods showed that the chromaffin cells fluoresce in a manner characteristic of the monoamine 5- hydroxytryptamine (5HT) rather than of the catecholamines (Kerkut, Sedden & Walker, 1967).

In addition Gaskell (1914) also reported that curare paralyzed the voluntary muscle system of Hirudo and both he and Pantin (1935) expressed the view that the excitation of the leech muscle was similar to that of vertebrate skeletal muscle. These observations led to the development of the well known method of bioassay for ACh by the

contemporary vertebrate physiologists and pharmacologists, using strips of the leech dorsal body wall (see McIntosh and Perry, 1950).

Thus to summarize the position at the middle of the 20th century, whilst it was fairly well established that chemical transmission was likely to be the mode of synaptic action in vertebrate peripheral nervous systems, with suggestions of similar peripheral mechanisms in some invertebrates, the electrical hypothesis of synaptic transmission in the vertebrate CNS continued to have a large measure of support.

However with the introduction of the intracellular microelectrode technique in experimental investigations of synaptic activity from 1951 onwards the final proof of chemical transmission at the vertebrate neuromuscular junction was obtained by Fatt and Katz (1951), at the smooth muscle junction by Bülbring (1955) and in the cat motoneurone by Brock, Coombs and Eccles (1952) and Woodbury and Patton (1952). Although the previous suggestions that ACh was likely to be a central transmitter were vindicated by the work of Eccles and his colleagues on the Renshaw cells of the spinal cord, it became clear that ACh was not the main transmitter within the vertebrate CNS (see Eccles, 1964).

With the advent of microiontophoresis (Nastuk, 1953) it became possible to examine the effects of brief applications of active chemicals on individual cells. This, coupled with the development of specific histochemical

methods (eg, Eränkö, 1955) have led to the general acceptance of the idea that amino acids and 5HT as well as the better known peripheral transmitters, ACh and the catecholamines, are probably transmitters at various vertebrate central synapses (see reviews by Eccles, 1964; Curtis & Crawford, 1969; Iversen, 1973; and Krnjević, 1974).

Application of these methods has led to parallel developments in studies of synaptic activity in the invertebrates, which has revealed a striking uniformity of certain substances used throughout the phyla where chemical transmission has been demonstrated with any certainty (see reviews by Florey, 1967; Welsh, 1972 and Gerschenfeld, 1973), as well as those found in the vertebrates; namely ACh, amino acids, catecholamines and 5HT.

In addition, just when it seemed that electrical synaptic transmission was about to be eliminated, the work of Furshpan and Potter (1959) using an invertebrate preparation (the crayfish abdominal nerve cord) provided conclusive evidence for the existence of an electrically operating synapse. Similar evidence for the vertebrate CNS (the Mauthner cells of the goldfish) was subsequentally provided by Furukawa and Furshpan (1963) and other electrical synapses have now been described at a variety of sites in both vertebrate and invertebrate nervous systems (see Bennett, 1972).

Thus the parallel developments in the study of synaptic activity in invertebrates brings us full circle from the early work at the start of the 19th century in the search

for analogous and possibly simpler systems to give us a better understanding of the complex processes underlying synaptic transmission.

The isolated innervated salivary gland of the cockroach may prove to be such a system.

## THE COCKROACH

The cockroaches were at one time included in the order Orthoptera, along with crickets, grasshoppers, mantids and stick insects (Imms, 1925). More recently (Imms, 1957) the cockroaches and mantids have been placed in a separate order, the Dictyoptera, with the cockroaches now forming the Suborder Blattaria, comprising some 450 genera and over 3,500 species.

The cockroach employed in this study, Nauphoeta cinerea (Olivier, 1789), was originally named Blatta cinerea by Olivier from a specimen taken in Mauritius and was transferred to its present genus in 1864 by de Saussure. It is commonly known as the lobster cockroach or the cinereous cockroach and is also known in Australia as the speckled cockroach (CSIRO, 1973). Rehn's (1945) study of the distribution of N. cinerea indicates that the native home of this species is East Africa and its wide occurrence throughout the tropical regions of both the Old and New Worlds is probably due to the activity of 16th century Arab and Portugese trading ships.

Nauphoeta cinerea (fig 1) is a large 'semi-domestic' species (25-29 mm long), of ashy colouring with a lobster like pattern on the pronotum; the wings of the male are slightly longer than the female and do not cover the abdomen. The development and reproductive characteristics of N. cinerea have been reported by Willis, Riser and Roth (1958). The species makes an ideal laboratory

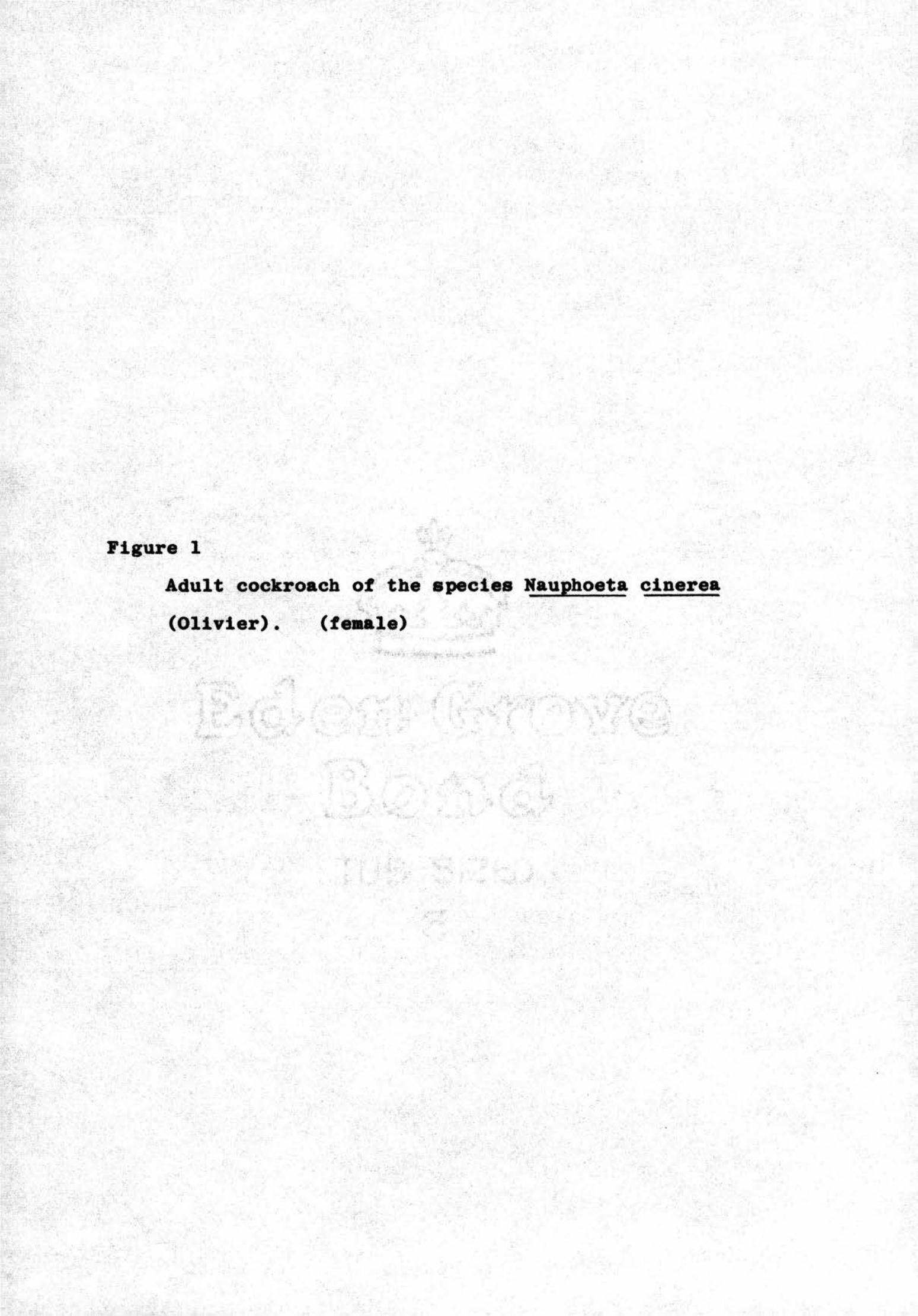
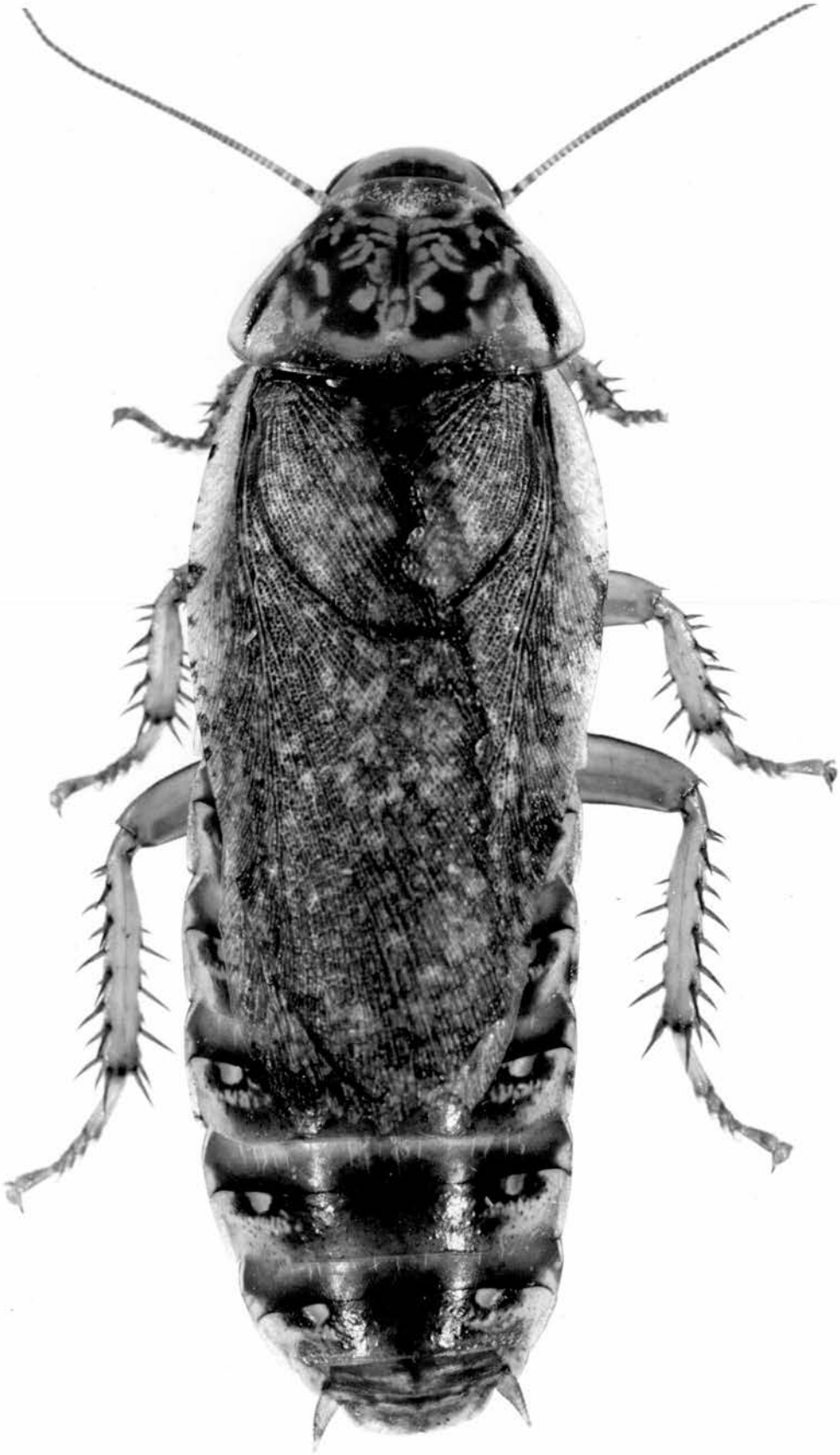


**Figure 1**

**Adult cockroach of the species Nauphoeta cinerea (Olivier). (female)**



subject since it is easily reared and has a low risk of infestation in the temperate latitudes.

'Of the other uses to which cockroaches have been put we have little to say. They constitute a popular remedy for dropsy in Russia and both cockroach-tea and cockroach pills are known in the medicinal practices of Philadelphia. Salted cockroaches are said to have an agreeable flavour which is apparent in certain popular sauces' - Miall and Denny (1886).

# SECTION I

# MORPHOLOGY

## INTRODUCTION

As stated in the general introduction much of the structure of the cockroach salivary system was described, at the level of the light microscope, by the end of the 19th century (e.g., Hofer, 1887; Lebedeff, 1899). More recently Day (1951), using similar methods, supported these findings. At the ultrastructural level both Kessel and Beams (1963) and Bland and House (1971) confirmed the earlier work, reporting that two cell types are found in the acini. The peripheral cells possess an intracellular ductule with large microvilli and numerous mitochondria, and are generally pyramidal in shape; the central cells have an extensive endoplasmic reticulum and large granules. In addition these authors found that the acinar cells were joined by septate desmosomes in their apical regions. From their structural studies Bland and House (1971) showed that the salivary glands of N. cinerea contained amylase, invertase, maltase and protease and suggested that these enzymes were secreted by the central cells whilst the peripheral cells transport ions and water.

With regard to the source and distribution of the innervation of cockroach salivary glands, this too was generally established by the latter part of the 19th century (e.g., Cholodkowsky, 1881; Hofer, 1887). However interpretation of the terminal structure was beset by the controversy on neuronal relationships prevailing in that period (see main introduction). The most

comprehensive modern study of the innervation of the cockroach salivary gland is that of Whitehead (1971) on P. americana. Whitehead showed that the gland was innervated from two sources, receiving axons from the sub-oesophageal ganglion and the stomadeal nervous system, both sets of fibres innervating the reservoirs, the salivary ducts and acini. Whilst this work confirmed the previous studies on the source and distribution of the innervation, Whitehead's ultrastructural studies provided no evidence for a distinctive junction such as those proposed by Kupffer (1874) and Hofer (1887). Terminals were found, however, that had shed their glial coats and contained electron dense vesicles clustered about a focus and in one case what appeared to be a 'synaptic plaque', lending support to the possibility of neuronal control by a chemical mediator. In addition Whitehead noted that these terminals were associated with either peripheral or central cells.

Although as a general rule the salivary glands of the Orthopteran and Dictyopteran insects receive a dual innervation, from the ventral nerve cord and the stomadeal nervous system, there is some variation in the exact origin and number of nerves emanating from these sources.

Cholodkowsky's (1881) comparative study of P. americana, B. orientalis and B. germanica showed that the innervation from the ventral nerve cord arises via a small pair of nerves on the posterior margins of the sub-oesophageal ganglion and passes to the glands via the reservoir ducts.

These observations were confirmed in B. germanica and B. orientalis by Hofer (1887) and in P. americana by Whitehead (1971). Nesbitt (1941) also described a similar arrangement in the nervous systems of seven of the nine families of Orthoptera and Dictyoptera included in his study. However he failed to find the same nerves in the cockroach Blaberus cranifer (Burm.); and in the stick insect Diapheromera femorata (Say) he noted the presence of two pairs of salivary duct nerves arising from this source.

The variation in the second source of the innervation appears to be its exact origin from the stomadeal nervous system. Miall & Denny (1886) described a small nerve emerging from what appears to be each of the posterior ends of the corpora allata in the retrocerebral complex of the stomadeal nervous system (Willey's nomenclature, 1961) to innervate the salivary glands of B. orientalis, an observation confirmed by Hofer (1887). A slightly different arrangement for the mantid (Mantis religiosa, L.), the cricket (Ceuthophilus brevipes, Scudder), and the grasshopper (Rhomalea microptera, Beauv.) was reported by Nesbitt (1941); in these insects the nerves arise from the hypocerebral ganglia of the retrocerebral complex. The salivary glands of the cockroaches P. americana and B. germanica receive their innervation from the stomadeal nervous system via fine lateral branches of the stomatogastric nerve on its course down the dorsal surface of the crop (Hofer, 1887; Willey, 1961; Whitehead, 1971).

Apart from Hofer's (1887) investigation of the tracts of the salivary nerves within the CNS no other studies appear to have been made to locate the cell bodies associated with the salivary nerves of the cockroaches.

The innervation of the salivary gland of the cockroach N. cinerea has been investigated by electrophysiological methods (House, 1973; Ginsborg and House, 1976), but no detailed anatomical study of its innervation has been reported. The gland has a racemose structure, but unlike its counterparts in vertebrates is not enclosed in a fibrous capsule. Thus the surface features of its acinar innervation are particularly amenable to investigation by scanning electron microscopy.

The present investigation was undertaken to provide a detailed account of its innervation to complement the electrophysiological evidence already available, provide a basis for further physiological studies and to make comparisons with the salivary glands of other species.

## METHODS

The salivary glands used in this study were dissected from the adult cockroaches (both sexes) of the species Nauphoeta cinerea (Olivier). The cockroaches were reared in glass aquaria at 22°C and fed rat cake and water ad lib.

Dissection.

The dissection was performed, with the aid of a dissecting microscope, under a standard physiological (cockroach) solution of the following composition: (mM): NaCl, 160; KCl, 1; $CaCl_2$, 5; $NaHCO_3$, 1; and $NaH_2PO_4$, 1; pH 6.9. (House, 1973)

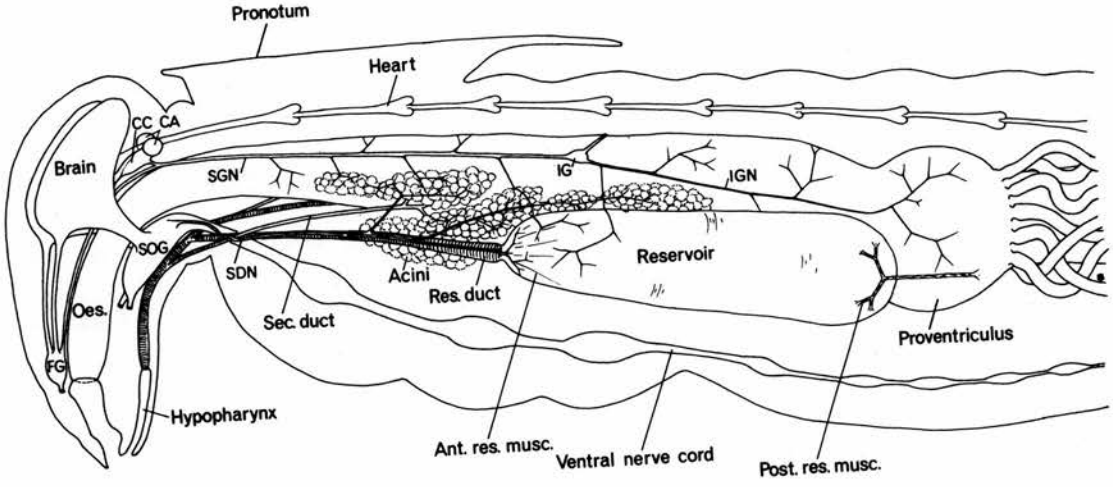
The complete salivary apparatus, comprising ducts, acini and reservoirs (fig 2) was removed by the following procedure. The insects were pinned down dorsal surface uppermost on a Sylgard resin (Dow-Corning) pad in a shallow perspex dissecting bath by pins through the base of the head and the tip of the abdomen, the latter being stretched slightly. No anaesthetic agent was employed but the insects were occasionally cooled to 4°C to restrict excessive activity. The wings, legs and antennae were removed and entrance into the abdomen was achieved by lifting the free edge of the 3rd or 4th abdominal tergum and cutting along the margins of the terga on either side as far as the pronotum. These were removed complete with the protonum using a needle to free any adhering tissue, revealing the underlying structures comprising the heart, (a tube lying along the median dorsal surface),

Figure 2

Diagram of sagittal section of the cockroach N. cinerea showing the principal features of the salivary gland, its innervation and associated structures.

Abbreviations:

| | |
|---|---|
| Ant. res. musc. | - anterior reservoir muscle |
| CA | - corpus allatum |
| CC | - corpus cardiacum |
| FG | - frontal ganglion |
| IG | - ingluvial ganglion |
| IGN | - ingluvial nerve |
| Oes | - oesophagus |
| Post.res. musc. | - posterior reservoir muscle |
| Res. duct | - reservoir duct |
| Sec. duct | - secretory duct |
| SDN | - salivary duct nerve |
| SGN | - stomatogastric nerve |
| SOG | - sub-oesophageal ganglion |


Pronotum
Heart
CC CA
Brain
SGN
IG
IGN
SOG
SDN
Acini
Res. duct
Reservoir
Oes.
Sec. duct
Proventriculus
FG
Hypopharynx
Ant. res. musc.
Ventral nerve cord
Post. res. musc.

close to their insertion on the posterio-apical margins of the SOG freed the salivary ducts and the SDN from their association with the ventral nerve cord; leaving the complete salivary gland attached to the crop. Dividing the posterior reservoir muscles and connective tissue strands joining the reservoirs dorsally, freed these structures from their attachment to the crop and proventriculus. By gently manipulating the reservoirs the gland was teased off the crop, starting at the posterior end and working forwards, and finally freed by cutting through the fine strands of connective tissue anchoring the anterior margins of the gland to the oesophagus. The intact gland was then transferred to a small petri dish and washed in several changes of cockroach saline.

## Light microscopy

Two basic histological methods have evolved to investigate the structure of the nervous system. One, the silver stain of Golgi was used to great advantage by Ramon Y Cajal to eludicate the structure of the vertebrate CNS, and the other the vital methylene blue stain of Ehrlich (1887) was similarly used by Zawarzin (1916) in his classic studies of the invertebrate CNS. Both methods are notoriously capricious. Since the actions of these compounds are not clearly understood and because the methods used lack any describable basis the techniques employed tend to be empirical. Methylene blue staining usually gives better results than silver impregnation in the invertebrates

(Bullock and Horridge, 1965).

a) Methylene Blue

Methods of applying this stain have varied from perfusion or immersion of tissues with simple solutions of the dye in water or in saline to more complex solutions containing buffers, anaesthetics, inhibitors of respiration and substances stimulating carbohydrate metabolism (Richardson, 1969). According to Nelemans and Dogteron (1953) each organ or tissue requires a "special prescription" for successful staining and there are differences between species which make it impossible to use the same solution indiscriminately.

Vital staining of the cockroach salivary gland was achieved by intra-abdominal injection of 0·1 - 0·2 ml of a 5% solution of methylene blue. The exact quantity injected being enough to distend the abdomen slightly. At periods of between 0·25 and 3h after injection, the glands were dissected out as described above. Tissues were either observed on cavity slides as wet preparations or stored overnight in 5% ammonium molybdate solution at 5°C. Following fixation with ammonium molybdate the glands were washed in saline, positioned on an albuminized cavity slide and the excess fluid withdrawn with an absorbent tissue. Keeping the slide horizontal the glands were dehydrated in a graded series of ethanols, cleared in xylene and mounted under a coverslip with Xam (Gurr's).

Solutions.

a) Methylene blue: This was prepared by dissolving 0·5 gms of crystalline methylene blue (Methelenblau med. pur. of Dr. G.G. Grübler & Co. Leipzig) in 100 ml of distilled water. The solution was heated and stirred until the solid was dissolved, filtered, cooled and stored at room temperature.

b) Ammonium molybdate: A solution saturated at 5°C was used initially, but a 5% (w/v) solution gave equally good results. The solutions were kept refrigerated and used cold.

b) Silver Staining

Use of Palmgren's (1948) method for the selective staining of nerve fibres and endings in mounted paraffin sections on the cockroach salivary glands failed to give clear results. Widespread deposition of a precipitate, containing both coarse and find granular material, proved difficult to eradicate and made it impossible to identify the fine acinar nerves. In addition non-specific staining of the secretory cells of the gland was hard to control. Various modifications were attempted to impregnate nerves in the intact gland; (e.g. Schofield's modification of Bielschowsky, see Drury and Wallington, 1967) these resulted in an absolute lack of differentiation between the nervous and non-nervous elements of the tissue. For these reasons, and because of the success obtained with methylene blue, experiments with silver stains were abandoned.

c) Acetylcholinesterase.

Since the observations by Bowser-Riley and House (1976) suggested a possible role for the neurotransmitter ACh in the cockroach salivary gland it was thought worthwhile to investigate the glands for the presence of the enzyme acetylcholinesterase.

Freshly dissected and unfixed salivary glands were mounted on albuminized cavity slides and incubated at 37°C for between 10 and 30 min in a 0·2% acetylthiocholine iodide (B.D.H.) substrate solution prepared as described by Page (1971). The preparation was then rinsed in two changes of distilled water, immersed in dilute ammonium sulphide (0·2 ml in 10 ml water) for 5 min, rinsed again in distilled water and finally dehydrated, cleared and mounted as described for the methylene blue procedure. The sites of cholinesterase activity appear brown while the background is pale yellow; 20 min incubation was found to give optimum colour development.

## Scanning Electron Microscopy

Following the introduction of commercially available scanning electron microscopes in 1965 a large number of papers have appeared which attest to the value of this instrument in biological research (see Johnson, 1976). The general principles of the technique have been described by Nixon (1971).

Many of the applications of scanning electron microscopy (SEM) to biological samples are dependent on the

adequate preservation of the material before its examination in the microscope column (Echlin, 1971). With respect to this, two basic problems in using this technique to study the surface features of the cockroach salivary gland had to be surmounted i.e., maintenance of the gross structural integrity of the intact gland and optimum fixation.

To maintain the structural integrity of the gland and minimize damage caused by handling the tissue throughout the lengthy preparative procedure the following method was devised. Salivary glands were mounted, under saline, in an open aspect (see fig 9d) on Sylgard resin (Dow-Corning) filled, stainless steel cups (fig 3a) and secured by stainless steel pins. (The overall dimensions of the cups were identical to those of the standard SEM specimen stubs). All subsequent operations were performed with the glands mounted in this way. During fixation and dehydration the cups were placed in the stainless steel holder (fig 3d), designed for ease of transfer between solutions contained in standard 200 ml laboratory beakers. An additional holder (fig 3c) was used to secure the cups during critical point drying (CPD), its shape and dimensions being dictated by the specimen tray of the CPD apparatus. Difficulties were encountered due to the loss of the resin pad from the smooth sided cup during this process and to overcome this the cup shown in fig 3b was employed. In addition, to minimize distortion of the Sylgard pad caused by the release of

Figure 3

Specimen handling system for scanning electron microscopy.

A. Stainless steel cup with smooth sided inner surface.

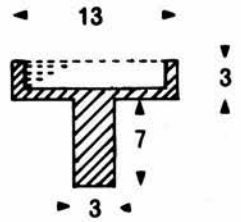
B. Stainless steel cup with undercut inner surface and central peg to prevent resin pad falling out during processing of specimens.

C. Cup holder for use with critical point drying apparatus.

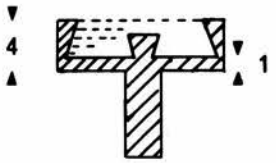
D. Cup holder for processing specimens in 200 ml beaker during fixation, dehydration etc.

Dimensions are given in mm.

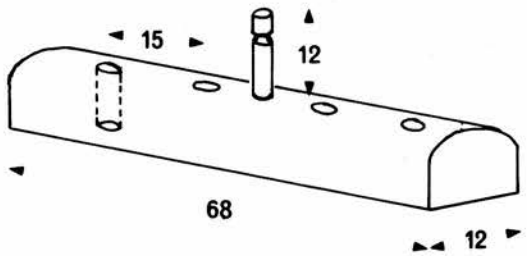
A



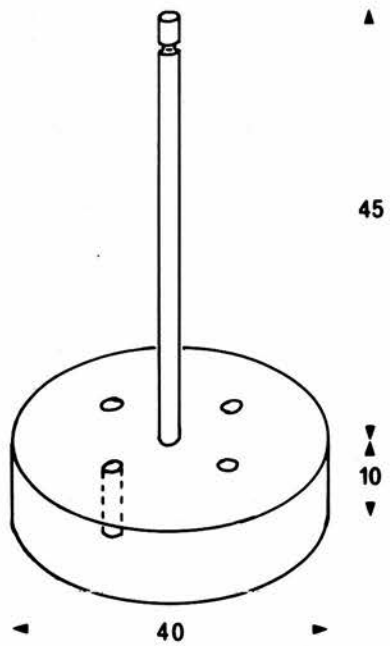

B




C




D



trapped air bubbles during the rapid pressure involved in the drying process, it was found necessary to degass the uncured resin prior to filling the cups.

Optimum fixation for SEM was obtained by immersion for one hour at room temperature in 2·5% glutaraldehyde with 8·5% sucrose in 0·05 M phosphate buffer pH 7·2 or 0·5% paraformaldehyde and 2% glutaraldehyde in 0·1 M phosphate buffer pH 7·2 (modified from Karnovsky, 1965). After fixation the tissue was rinsed in several changes of phosphate buffer, dehydrated to absolute ethanol, and taken in to acetone prior to drying at the critical point of liquid $CO_2$. This was achieved by transferring the tissues, under acetone into the specimen holder (fig 3c) which was then quickly placed in the specimen chamber of the CPD apparatus. The chamber was immediately sealed and liquid $CO_2$ allowed to rapidly cover the specimens. Numerous soaks and flushings (draining to just above the specimens) were carried out over a period of 4 - 5 hrs. This was done to insure a complete removal of acetone and complete saturation of the glands with liquid $CO_2$. The temperature of the chamber was then gradually raised over a period of about 30 mins to 45°C. The chamber pressure, previously kept at about 1300 psi was then bled very slowly over a period of 30 mins. The specimens were then removed from the chamber and placed in a vacuum evaporator and coated with gold-palladium before examination on a Cambridge Stereoscan microscope at an accelerating voltage of 10 kV.

HCl-collagenase digestion of the basement membrane was achieved with the method of Evan, Dail, Dammrose and Palmer (1976). Tissues were fixed for three hours (instead of one) in the modified Karnovsky fixative described above, rinsed in phosphate buffer (0·1 M, pH 7·2) and immersed in 8N HCl for one hour at 60°C. After HCl digestion tissues were again rinsed and placed in phosphate buffered collagenase (Sigma, type II) pH 7·2, at a concentration of 1 mg/ml of buffer, for no more than four hours at 37°C. Tissues were postfixed for 30 min, dehydrated and prepared for SEM as specified above.

The most critical part of the HCl-collagenase digestion procedure was found to be the treatment with collagenase. The process of digestion could only be monitored visually at this stage and the incubation terminated for individual preparations depending on the degree of dissolution of the tissue. A minimum of 2 h incubation was necessary for any significant digestion of the basement membrane to appear upon examination by SEM. Long periods of digestion (in excess of 4 h) resulted in complete dissolution of the tissue.

## Intracellular staining

Prior to 1968 there were only two histological methods for the selective and complete staining of single neurones. These were the Golgi and methylene blue methods. Following the publication by Stretton and Kravitz (1968) of a detailed method for staining individual living nerve cells

with Procion dye it became clear that a near perfect method for the identification of individual nerve cells had been found. The method consisted of iontophoretically injecting Procion yellow from a micropipette into the living nerve cell of the lobster abdominal ganglia. On subsequent fixation and examination by fluorescent microscopy it was seen that virtually the entire cell, including its fine processes, had been filled with the dye. This method was rapidly adopted to investigate identified neurones in both vertebrate and invertebrate nervous systems (see Kater and Nicholson, 1973). A modification of this technique was devised by Iles and Mulloney (1971) to identify cockroach motoneurones by passing the dye through the cut end of the peripheral nerve.

In this study the innervation of the salivary gland was examined using a modification of the axonal filling method of Iles and Mulloney (1971), employing cobalt chloride (Pitman, Tweedle and Cohen, 1972) and horseradish peroxidase (Graham and Karnovsky, 1966) as intracellular markers. These were chosen because of their high mobility in the absence of applied current (see Lavail and Lavail, 1972 and Tyrer and Altman, 1974), the application of which may produce less specific filling and apparently some artifacts (Tyrer and Altman, 1974). Tyrer and Altman suggested that cobalt moves up the axons by simple diffusion. Another possible mechanism may involve currents generated by the cut nerves as a result of the demarcation, or injury potential (Kater, Nicholson & Davies, 1973).

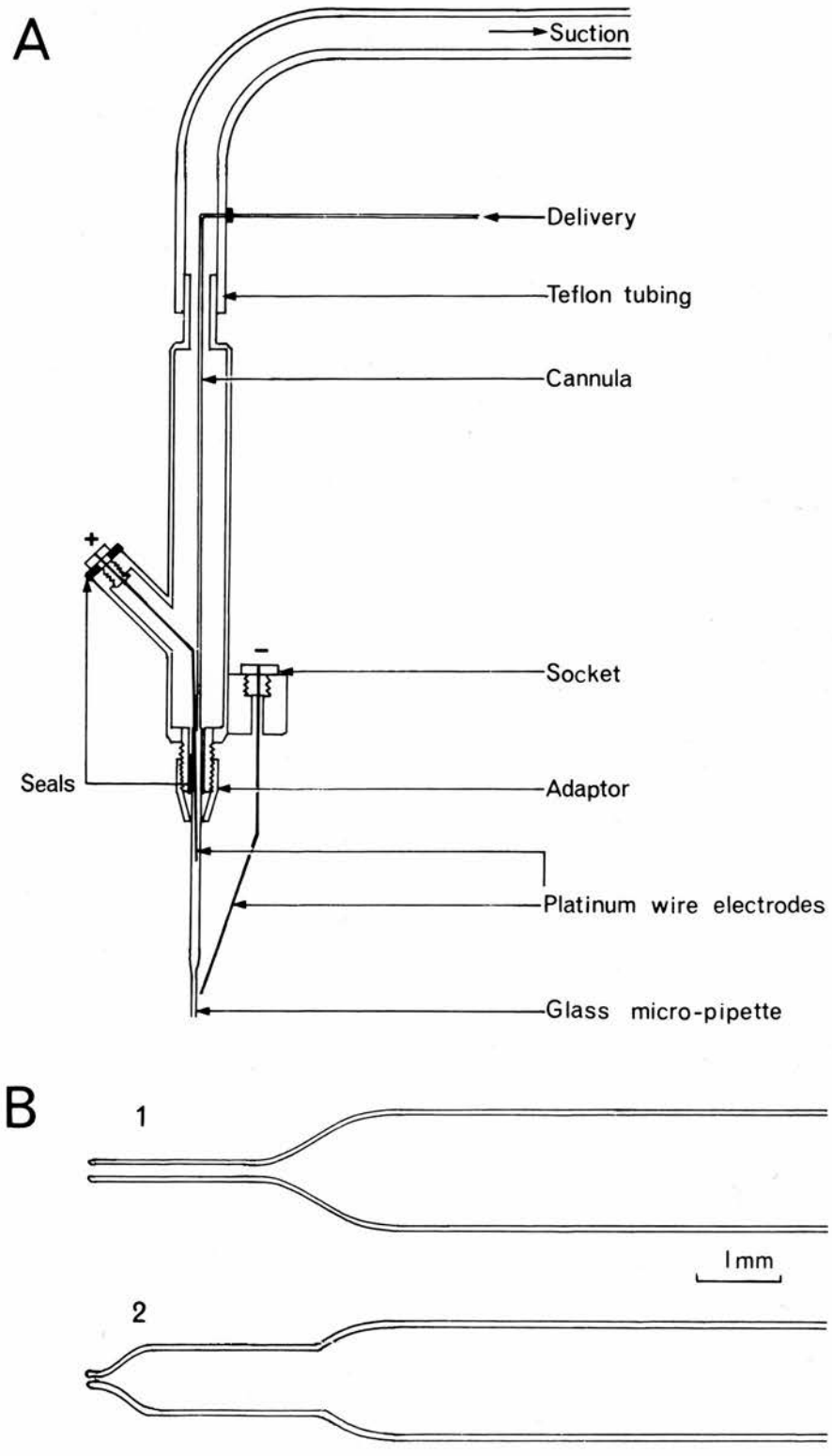
Lavail and Lavail's results suggested that an active axonal transport mechanism was responsible for the intracellular movement of HRP.

The main purpose in employing this method was to ascertain the location within the cockroach CNS of the cell bodies associated with the salivary duct axons; however, it was apparent that the same technique could be employed to investigate the distribution of these axons within the gland. The experimental approach was the same in each case. The salivary glands and sub-oesophageal ganglion (SOG) were exposed by dorsal dissection (as described above) and left in situ with the ducts and nerves intact. The crop, with the attached gland was deflected to one side and the contralateral duct nerve identified. The salivary duct nerve (SDN) was cut close to its insertion on the salivary duct (or SOG), leaving a stump of 100 - 150 $\mu$m long between the cut end and its insertion into the SOG (or gland). The cut end of the SDN was drawn into a suction device (fig 4a), the tip diameter of the micropipette (fig 4b) previously adjusted to ensure a tight fit around the nerve (see figure legend for details). Backfilling the micropipette with either HRP or cobalt solutions exposed the cut end of the nerve to the appropriate solution. Use of this device effectively isolated the rest of the tissue from non-specific uptake of the axonal dyes. The preparation was perfused at room temperature with the cockroach saline employing a Watson Marlow H.R. flow inducer set to deliver 5 ml/min for periods of between

Figure 4

Suction device/stimulating electrode.

This dual purpose apparatus was employed in electrophysiological experiments to stimulate the salivary nerves and in the morphological experiments to apply intracellular markers. The adaptor allowed easy interchange of glass micro-pipettes (B; 1.2) to suit the requirements of individual experiments. The micropipettes were formed from standard (2 mm) microelectrode glass on a vertical electrode puller (Forth Ins.), and cut to give the diameter required to ensure a good fit around the ducts or nerves. By lowering the tip into the heated coil of the puller the cut edges of the glass were 'polished' and a fine adjustment of the diameter achieved. The conformation of the pipette shown in B1 was employed to stimulate the salivary nerves, the long parallel shank being required to draw up a good length of nerve. That shown in B2, obtained by double pulling, was employed to apply intracellular markers to the cut end of the nerve. Backfilling of the micro-pipette was achieved using a micro-syringe connected to fine polythene cannula tubing sealed into the teflon suction tube. The broad shank of the pipette was found necessary to allow the cannula to come close to the tip and ensure rapid diffusion of the markers to the cut end of the nerve.


A
Suction
Delivery
Teflon tubing
Cannula
+
−
Socket
Seals
Adaptor
Platinum wire electrodes
Glass micro-pipette
B
1
1mm
2

3 and 12 hr. Cobalt chloride was prepared as a simple solution at a concentration of 500 mM. HRP (Sigma, type IV) was prepared in a 0·1 M phosphate buffer pH 7·6 of a concentration of 10 mg/0·1 ml. Pretreatment of the nerve stump with distilled water for 10 - 15 min (via the suction device) to osmotically swell the cut ends of the axons (Kater et al, 1973) produced more consistent fillings. At the end of the filling period the SDN was cut as close to the micropipette as possible and the preparation dissected away from the surrounding tissue. The presence of cobalt and HRP was detected by the methods of Pitman, Tweedle and Cohen (1972) and Graham and Karnovsky (1966) respectively; the contralateral unfilled SDN in each preparation served as a control.

For cobalt filled axons the preparations were immersed for 20 - 30 min at room temperature in 10 ml of insect saline containing approximately 0·05 - 0·1 ml of 100% ammonium sulphide solution. The preparation was then washed in saline and fixed for 1 hour in a modified Karnovsky fixative (as for SEM) at pH 7·2. After fixation the material was dehydrated to absolute ethanol, cleared in creosote and viewed as a whole mount on a cavity slide. The dehydrating alcohols were buffered with 0·15 M phosphate buffer (pH 7·2) to prevent loss of staining precipitate which is soluble in acid (Pitman, et al, 1972). Cobalt filled axons appeared black on a light yellow background.

The presence of HRP was demonstrated by immersion of

the fixed tissue (1 hr in the modified Karnovsky as above) in a solution of 3-3' diaminobenzene (100 mg DAB in 200 ml 0·1 M Tris/HCl buffer, pH 7·6 + 0·8 ml $H_2O_2$*) at 37°C for periods of between 0·5 - 2 hrs. Preparations were then washed in phosphate buffer (0·1 M) and dehydrated to absolute ethanol, cleared in xylene and viewed as whole mounts on cavity slides. HRP filled axons appeared dark brown on pale unstained background.

Both staining reactions were visually monitored at regular intervals, the incubation being terminated when maximum development of each stain had occurred.

* N.B. DAB solution prepared and reaction performed in a fume cupboard with rigid precautions against any form of bodily contact because of its known carcinogenic properties, and has now been withdrawn and replaced by 3-amino-9-ethyl carbazole.

# RESULTS

## Light Microscopy

The paired salivary glands of *N. cinerea* are composed of acini, ducts and reservoirs, lying within the thorax between the ventral nerve cord and the crop (fig 2). The acini on each side are arranged in groups or lobes around the terminations of a branched duct system, which converges into a common secretory duct separately joining the main salivary duct. The main salivary duct; formed by the fusion of the two reservoir ducts opens on the labium at the base of the hypopharynx (fig 2). The manner in which the acinar ducts and reservoir ducts join to form the common salivary duct is shown in fig 5a. The fine structure of the acini and secretory ducts has been described by Bland and House (1971) and the structure and function of the reservoirs by Sutherland and Chillseyzn (1968). The gland receives an innervation from two sources, the ventral nerve cord and the stomatogastric nerve similar to that described for *P. americana* by Whitehead (1971).

Light microscopic examination of methylene blue stained glands showed that the innervation from the ventral nerve cord is a pair of nerves arising from the posterio-apical regions of the sub-oesophageal ganglion, which pass on to the gland via the lateral margins of the salivary ducts (fig 5b). At this point the nerves either run directly on to the reservoir ducts or join the secretory ducts for a short distance before crossing to the reservoir

Figure 5

A. Unstained light micrograph showing the arrangement of the salivary ducts. CSD common salivary duct; Res D reservoir duct; Sec D secretory duct. B - F, light micrographs of methylene blue stained preparations.

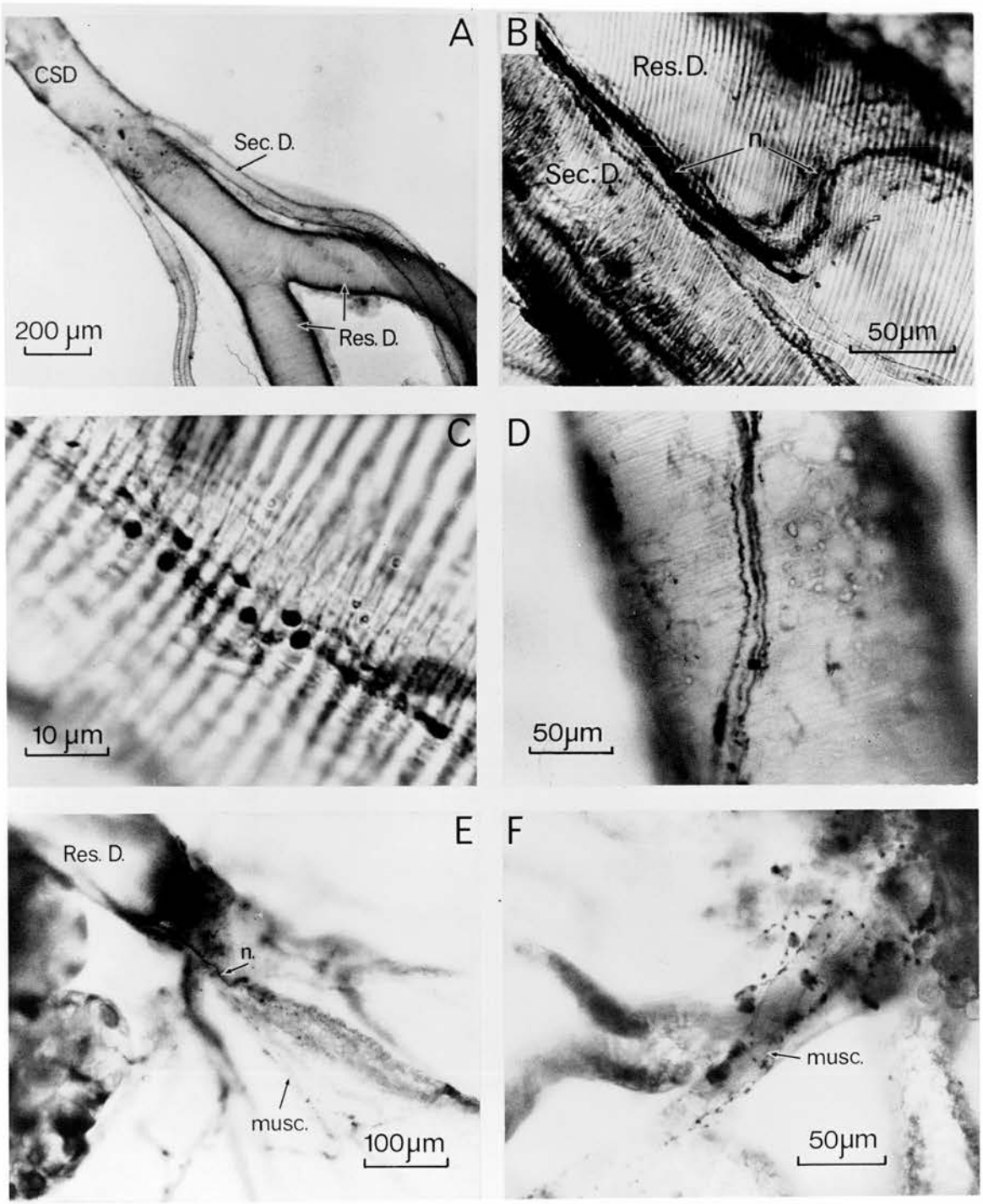
B. Salivary duct nerve just posterior to the point where the secretory ducts fuse with the reservoir ducts. In this case the nerve (n) becomes associated with the secretory duct (Sec. D.) for a short distance before crossing to the reservoir duct (Res. D.). Note striated structure of the ducts.

C. The two larger axons of the salivary duct nerve exhibiting irregularly spaced swellings.

D. Salivary duct nerve mid-way down the reservoir duct. Note three distinct groups of axons.

E. Final branch of the salivary duct nerve (n) supplying the muscles at the mouth of the reservoir.

F. Network of fine beaded axons on the reservoir muscles (musc.) formed by the final branch of the salivary duct nerve.


A
CSD
Sec. D.
Res. D.
200 µm
B
Res. D.
n.
Sec. D.
50µm
C
10 µm
D
50µm
E
Res. D.
n.
musc.
100µm
F
musc.
50µm

duct (fig 5b), which they follow on their main course into the gland.

Each duct nerve is initially composed of two large axons about 3 $\mu$m diameter and several smaller axons of less than 1 $\mu$m (fig 5b). The larger axons occasionally exhibit 3 - 5 $\mu$m swellings along their length (fig 5c). The composition of the duct nerves has been confirmed by transmission electron microscopy (House, 1977). As the nerves pass down the ducts they divide and form two main branches which run on either side of the ducts. Each branch is composed of several axons (fig 5d); subsequent branches of these axons leave the duct (fig 6a) to form the acinar nerves. The first branch usually passes forward to supply anterior groups of acini, the more distal branches supplying both medial and lateral acinar lobes. This is in contrast to P. americana whose duct nerves are relatively free of branches until they reach the reservoir mouth (Whitehead, 1971). The lateral acinar lobes are supplied by two or more branches of the ipsilateral duct nerve, whereas the central lobes receive branches from both left and right duct nerves. The final branch of the duct nerve (fig 5e) forms a network of fine beaded axons on the musculature at the mouth of the reservoir (fig 5f). Contraction of these muscles has been observed following electrical stimulation of the duct nerve.

Upon reaching the acinar surfaces the nerves undergo profuse branching forming a complex network or plexus of fine axons (figs 6; b,c,e) with diameters of less than

Figure 6

A - D, light micrographs of methylene blue stained preparations.

A. Lateral branch of the salivary duct nerve (SDN) leaving the reservoir duct (Res. D.) to innervate acini (Ac.) via fine beaded axons (Ax.).

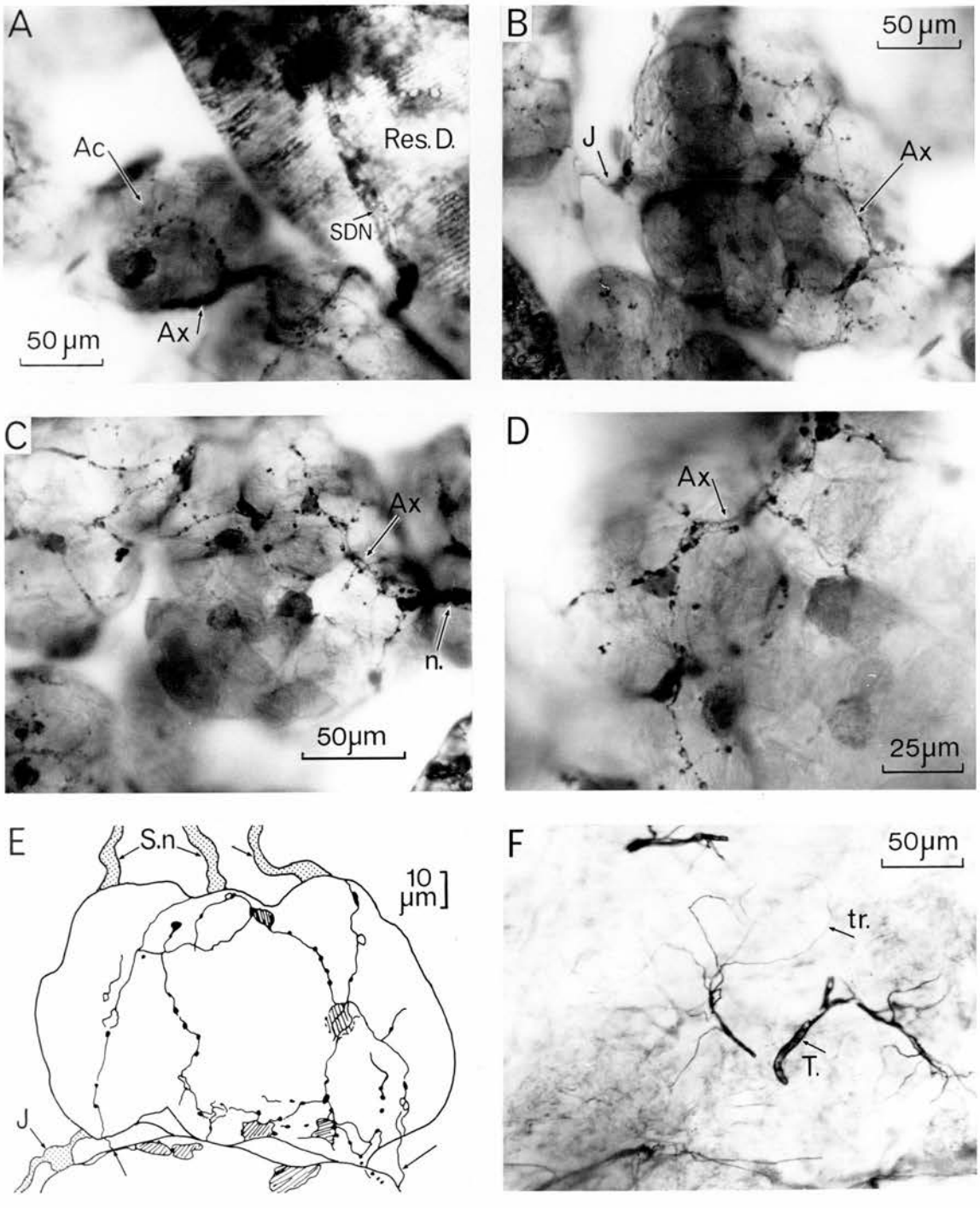
B. Fine beaded axons on acinar surface with complex junction (J) between at least two other adjacent acini.

C. Group of acini with large branch of duct nerve at right (n.), forming a neural plexus on the acinar surface. Note axonal swellings and the presence of at least two axons traversing the surface together.

D. Higher magnification micrograph of multiple axon (Ax.) shown in figure C.

E. Camera lucida reconstruction of another methylene blue stained acinus showing an extensive network of beaded acinar axons. Note contact with adjacent lower acinus (arrowed), the input of three sheathed duct nerves (S.n and stippled) and complex junction (J and stippled) off acinar surface. Darker areas (hatched in drawing) are the stained nuclei of acinar cells.

F. Branching pattern formed by the fine terminations of the tracheal system within the acini. T - trachea; tr. tracheoles. Unstained preparation, phase contrast.


A
Ac
Res. D.
SDN
Ax
50 µm
B
50 µm
J
Ax
C
Ax
n.
50µm
D
Ax
25µm
E
S.n
10 µm
J
F
50µm
tr.
T.

1 $\mu$m. The axons of this plexus run either singly or in groups of two or three over the surface (figs 6; c,d) forming connections with adjacent acini (figs 6; b,e). In this way the plexus is extended over each acinar lobe giving the appearance of a closed network. In addition some of the acinar axons form connections with the plexuses of neighbouring lobes (fig 7d) and small secretory ducts (fig 7c).

The apparent closed network formed by the acinar axons contrasts with the dendritic branching pattern formed by the fine terminations of the tracheal system (fig 6f). This arises from within the acini via the relatively larger trachea which follow the course of the secretory ducts.

The acinar axons exhibit swellings at irregular intervals (figs 6; b-e) similar in size to those reported in this and other salivary glands (see Table 1). Similar structures have been reported in the vertebrate autonomic nervous system (e.g. Gabella, 1976) and have been called variously swellings, beads, enlargements or varicosities. In this thesis the term axonal swelling will be used. Particularly clear examples of the axonal swellings on the acinar surface are shown in figures 7; a,b, and may be compared to those seen by scanning electron microscopy (see later figs 13; c,d).

The acinar innervation seen in glands stained with methylene blue corresponds to that observed by Bland et al. (1973) in glands treated by the Falck-Hillarp method for

TABLE 1

Axonal swellings in insect salivary glands

| SPECIES | THICKNESS OF AXONAL SWELLINGS | METHOD | REFERENCE |
|---|---|---|---|
| *Nauphoeta cinerea* (Oliv.) | Mean 1·4 μm<br>Range 1·0 - 3·0 μm | M.B. | Present study |
| | Mean 1·5 μm<br>Range 1·1 - 1·9 μm | SEM | Present study |
| | Mean 1·9 μm<br>Range 1·1 - 2·9 μm | FLUOR | Bland et al. (1973) |
| *Periplaneta americana* (L.) | Mean 2·1 μm<br>Range 1·1 - 4·2 μm | M.B. | Whitehead (1971) |
| *Schistocerca gregaria* (Forskål) | Mean 1·8 μm<br>Range 1·0 - 4·00 μm | FLUOR | Klemm (1972) |

Abbreviations: M.B. methylene blue;

SEM. Scanning electron microscopy;

FLUOR. technique of Falck for formalin induced fluorescence.

Figure 7

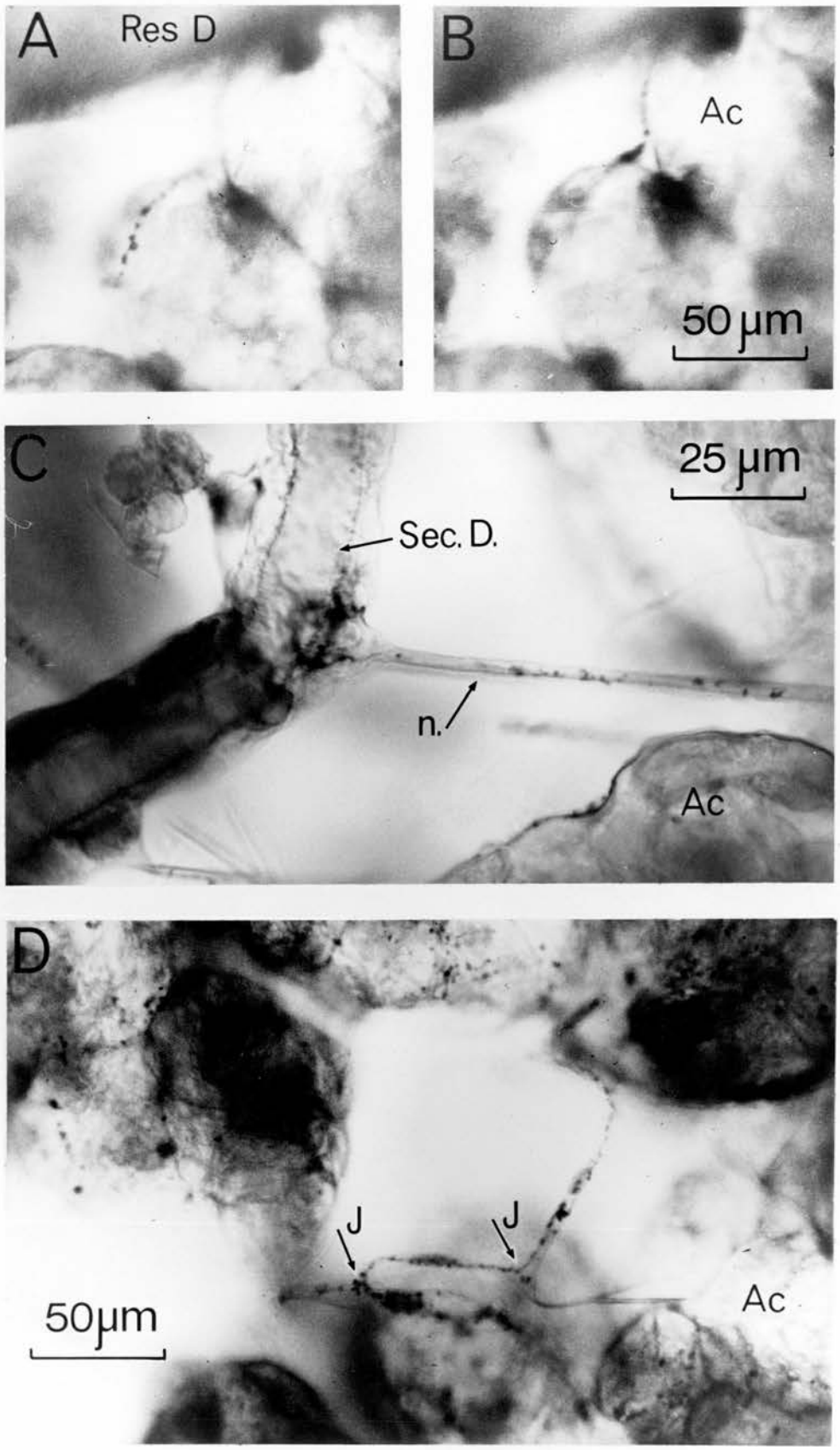
Light micrographs of methylene blue stained glands.

A. Acinar axon exhibiting irregularly spaced swellings running along the edge of an acinus.

B. Continuation of the axon shown in fig A. seen in different focal plane.

C. Acinar nerve (n) associated with a small secretory duct (Sec. D).

D. Neural junctions (J) between adjacent acinar groups.


A
Res D
B
Ac
50 µm
C
25 µm
Sec. D.
n.
Ac
D
J
J
50µm
Ac

monoamines. Application of catecholamines to the gland mimics the electrical responses recorded from the acini following stimulation of the duct nerves (House et al, 1973). In a further series of experiments (Bowser-Riley & House, 1976) to test the actions of other known transmitter substances on the gland, acetylcholine and carbachol appeared to act by modifying transmitter output from the salivary nerves. It was therefore thought worthwhile to investigate the innervation of the gland by a thiocholine cholinesterase technique (Page, 1971) for the detection of acetylcholinesterase (AChe). In five glands investigated using this method no AChe could be detected in the acini and duct nerves. However the stomatogastric nerve and its associated branches stained dark brown indicating that high levels of the enzyme were present.

The stomatogastric nerve is part of the stomodeal nervous system of the cockroach and has been well described by Willey (1961). It originates in the frontal ganglia and passes down the dorsal surface of the oesophagus and crop, slightly to the right of the midline forming the ingluvial ganglion midway between the head and the proventriculus (fig 2). Two nerves, the ingluvial nerves, arise from this ganglion and run on either side of the crop terminating in the region of the proventriculus. Principally the stomatogastric and ingluvial nerves innervate the crop, but also give rise to several fine lateral branches that appear to be associated with the gland. It proved difficult to follow the more anterior branches

of the stomatogastric nerve (SGN), since they become enmeshed with the more numerous crop branches and the connective tissue between the gland and the crop. However, some of the posterior branches of the SGN and anterior branches of the ingluvial nerves were traced to acini lying adjacent to the reservoirs, but apparently did not form acinar plexuses similar to those originating from the axons of the duct nerve.

Other branches of the stomatogastric and ingluvial nerves were found to be continuous with the axons of densely staining multipolar neurones that were confined to the anterior regions of the reservoirs (figs 8; a-e). The dendrites from these cells branch profusely giving rise to a plexus of fine neural processes which have a characteristic beaded appearance (figs 8; c,d). The plexus forms a delicate interwoven meshwork that runs over the anterior surfaces of the reservoir. The morphology of these cells bears a striking resemblance to those reported as sensory neurones in the abdomen of the blowfly larva by Osborne (1963).

Figure 8

Light micrographs of methylene blue stained glands.

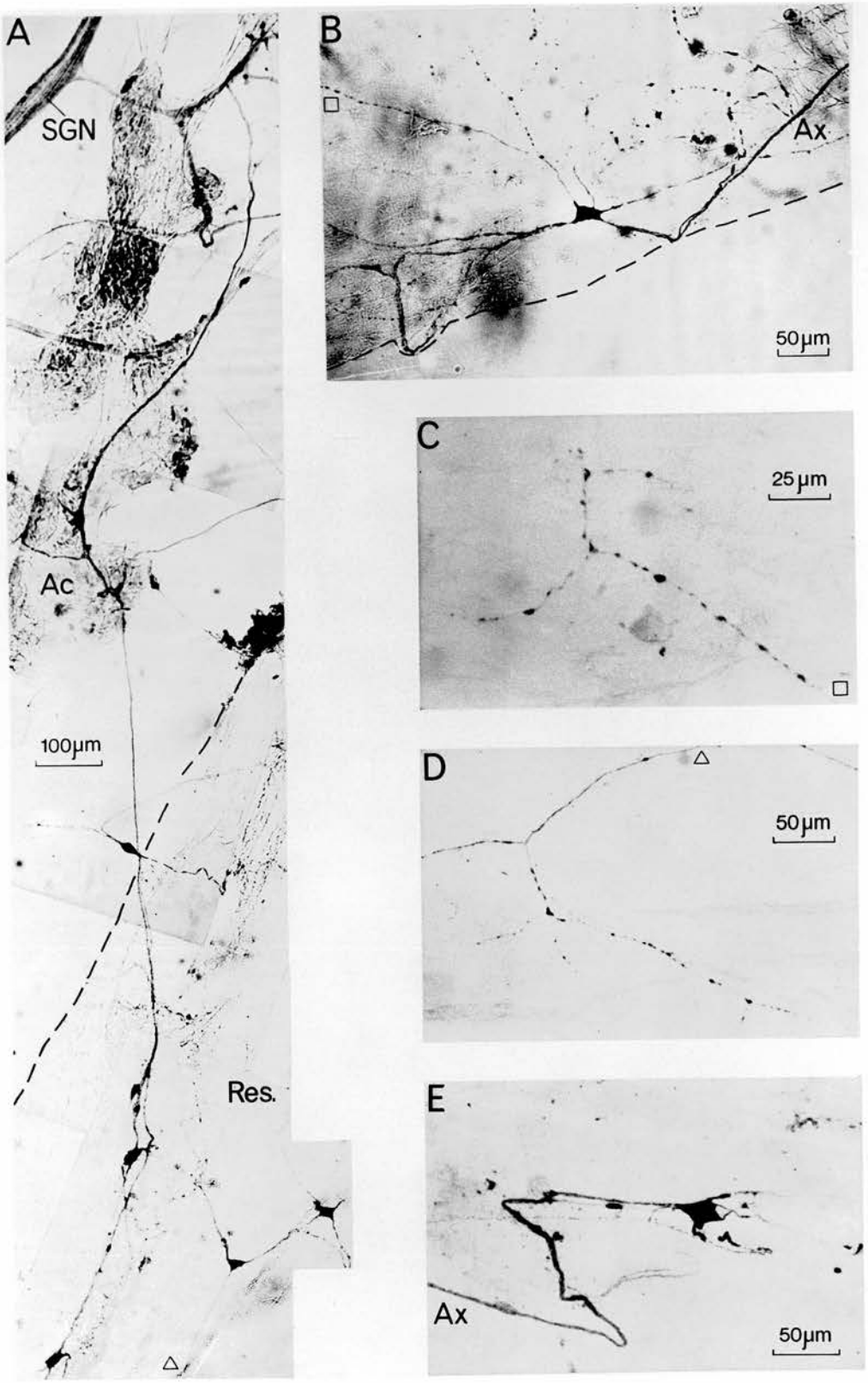
A. Micrograph montage showing the association between the stomatogastric nerve (SGN) and the group of interconnected densely staining neurones lying on the reservoir (Res). Note apparent association between the axons and acini (Ac).

B. Densely stained neurone lying on the border of reservoir (dotted line). Note long axon (Ax) and dendritic projections ramifying over a considerable area of reservoir surface.

C. Detail of fine beaded plexus formed by dendritic processes from cell marked □ in figure B.

D. As above for process marked △ in figure A.

E. Densely stained neurone lying centrally on the reservoir surface.


A
SGN
Ac
100µm
Res.
B
Ax
50µm
C
25µm
D
50µm
E
Ax
50µm

Scanning Electron Microscopy.

The extent of the neural network shown on the acinar surfaces by methylene blue cannot be fully determined with the light microscope, because many of the fibres are at the limits of its resolution, it was therefore thought worthwhile to investigate the surface features of the gland by scanning electron microscopy (SEM). The cockroach salivary gland, unlike its counterparts in vertebrates is not enclosed in a fibrous capsule, thus its surface features are particularly amenable to investigation by this method.

Scanning electron micrographs of the gland (fig 9; a,d) revealed the same gross structure as that observed with light microscopy. The well defined acini (100 - 150 $\mu$m in diameter), exhibit an irregular spherical appearance, their surface irregularities probably being due to the arrangement of the underlying cells. The acini are arranged in groups around the terminations of secretory ducts (fig 9 b); the branching pattern of these ducts is evident only when viewing the gland on its dorsal surface, i.e. the surface that lies adjacent to the crop. However, glands were usually scanned on the opposite or ventral surface to facilitate tracing the acinar axons where they are more numerous.

This became apparent from observations made on the dorsal surfaces during a course of experiments to trace the branches of the stomatogastric nerve associated with the acini seen in methylene blue stained glands. Because

Figure 9

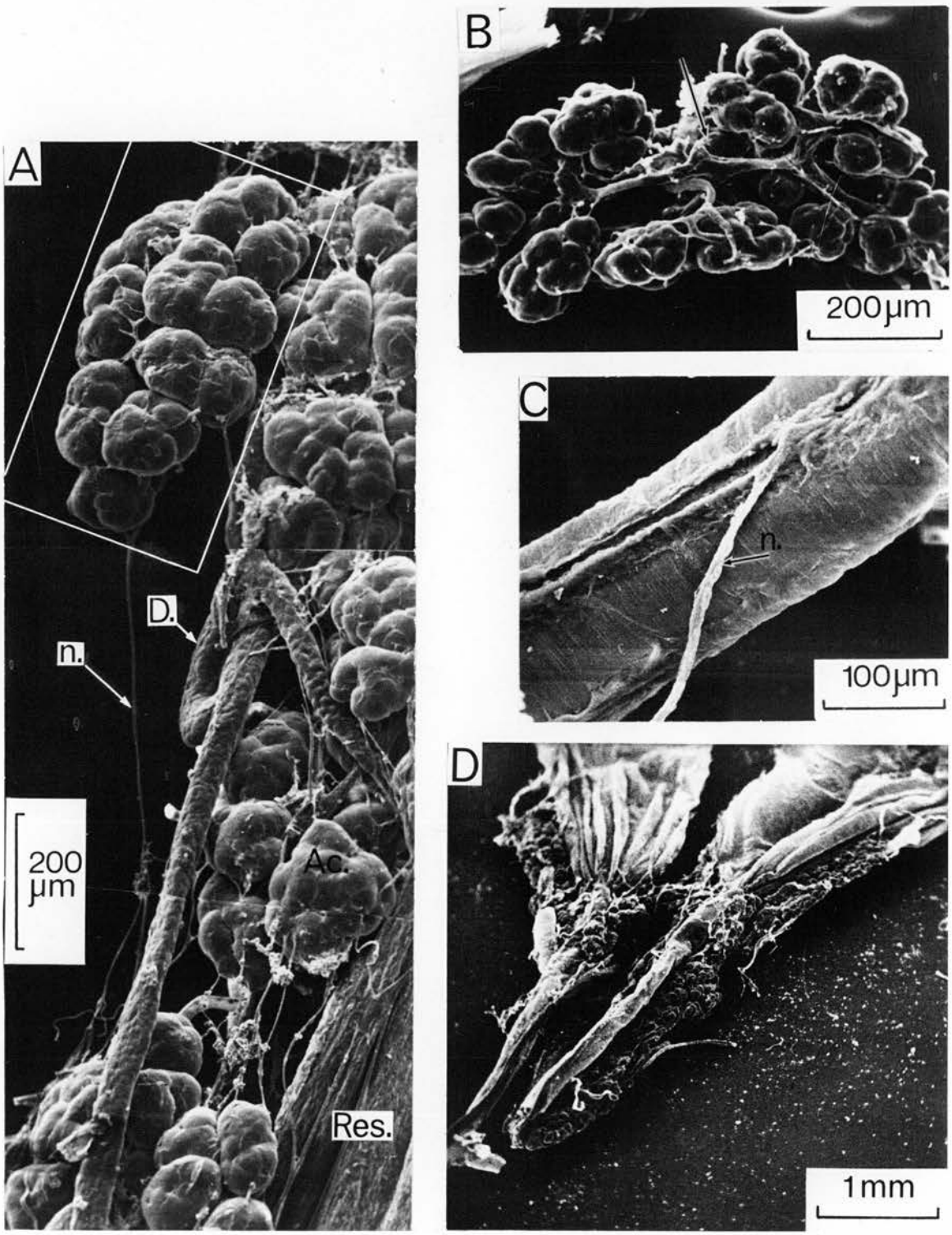
Scanning electron micrographs of normal salivary glands.

A. Micrograph montage showing acini (Ac), nerves (n), secretory ducts (D) and part of a reservoir (Res).

B. Isolated group of acini viewed from their ventral surface. Note ductal tree converging to small secretory duct marked by arrow, which passes out between the acini on to the dorsal surface.

C. Nerve trunk (n), displaced from connective tissue trough in its course down the reservoir duct. Note striations of duct and connective tissue anchors passing around the duct.

D. Low power view of salivary gland, seen from its dorsal surface with the reservoirs in the upper part of the micrograph, showing how the preparation was arranged on Sylgard resin filled cups.


A
D.
n.
200
µm
Ac.
Res.
B
200µm
C
n.
100µm
D
1mm

of the complex arrangement of the SGN (see fig 2) it was not possible to obtain preparations with intact branches that could be traced to any part of the gland.

The duct nerves (fig 9c) run down the ventral surface of the ducts following a similar course to those seen in methylene blue stained glands. These nerves lie within a channel or trough formed by connective tissue which is firmly attached to the surface by anchors passing around the ducts, the whole structure serving to hold the nerve trunk on to the duct surface. In addition the duct nerves and their branches are enclosed in a substantial sheath which at its junction with the acinar surface becomes continuous with the basement membrane (see fig 10, 11a).

A striking feature of the electron micrographs is the richness of the acinar innervation. This is clearly seen in figure 10a which is a montage constructed from a series of higher magnification micrographs of the group of acini outlined in figure 9a. The acinar axons can be traced as surface ridges under the basement membrane and form a similar plexus to that observed with light microscopy. (Sections of acini observed with transmission electron microscopy also exhibit axon-containing protrusions of the basement membrane of similar dimensions, D.J. Maxwell, personal communication). Figure 10b, a tracing of figure 10a, shows part of the extensive innervation of these acini, recourse being made to other micrographs taken from different angles and at higher magnifications to

Figure 10

A. This figure is a composite of numerous micrographs taken at a higher magnification of the group of acini outlined in figure 9A.

B. The overlay is a tracing of this composite and shows part of the extensive innervation of these acini. Note multiple input to this acinar group, extensive surface innervation and nerve associated with secretory duct (lower right). Pathways of acinar axons not established with certainty, because of limitations set by the geometry of the preparation, are indicated by broken lines.

confirm their presence (for example see figs 11 & 12). However the pathways of all the axons could not be established with certainty because of the limitations set by the geometry of the preparation. Evidently the axons traverse the acinar surface for some considerable distance before passing on to adjacent acini.

Connections between adjacent acini are numerous and can be considered as either simple or complex. The simple junctions are more numerous and consist of single connections between adjacent acini (figs 12; a,c); the complex junctions have the appearance of 'ganglia-like' structures (fig 12b) and may connect as many as five acini within any one group. Multiple connections are also made between adjacent acinar groups and occasionally acinar axons become associated with small secretory ducts (fig 10). (for similar examples in methylene blue stained glands see previous figs 7; c,d).

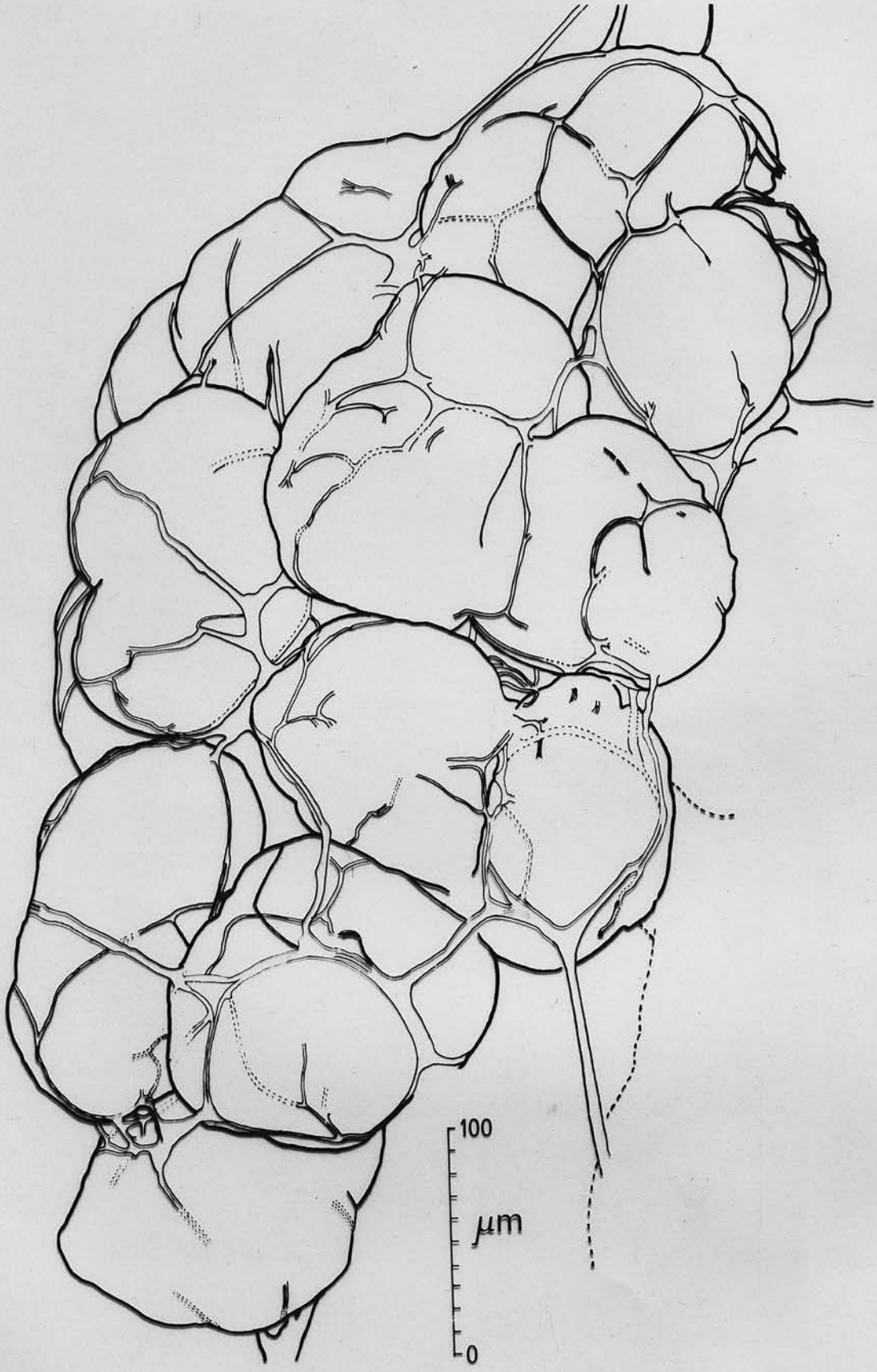
At higher magnifications some of the acinar nerves appear as tracts of up to four units (figs 11; a - f). Although the number of axons could not be estimated with precision it certainly seems obvious that the acini receive multiple innervation.

Axonal branching occurs not only between adjacent acini but also on the acinar surface forming complex patterns and junctions (fig 11; e,f); in addition acinar axons could occasionally be seen to cross (fig 11; c,d).

Occasionally some of the nerves on the acinar surface were observed to exhibit an irregular beaded appearance

A
B

B



A

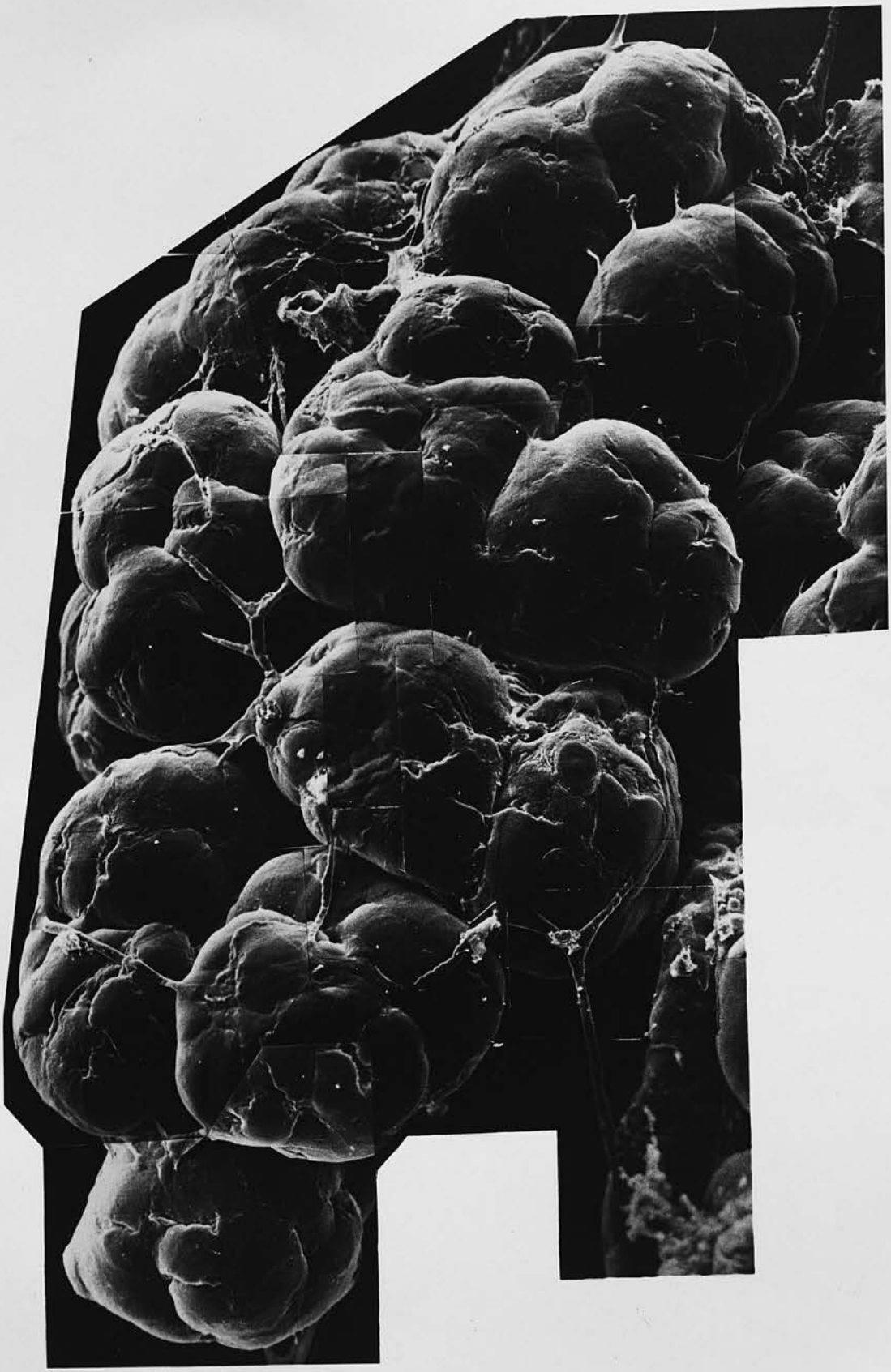


Figure 11

Scanning electron micrographs of normal salivary glands.

A. Complex innervation pattern on acinar surface.

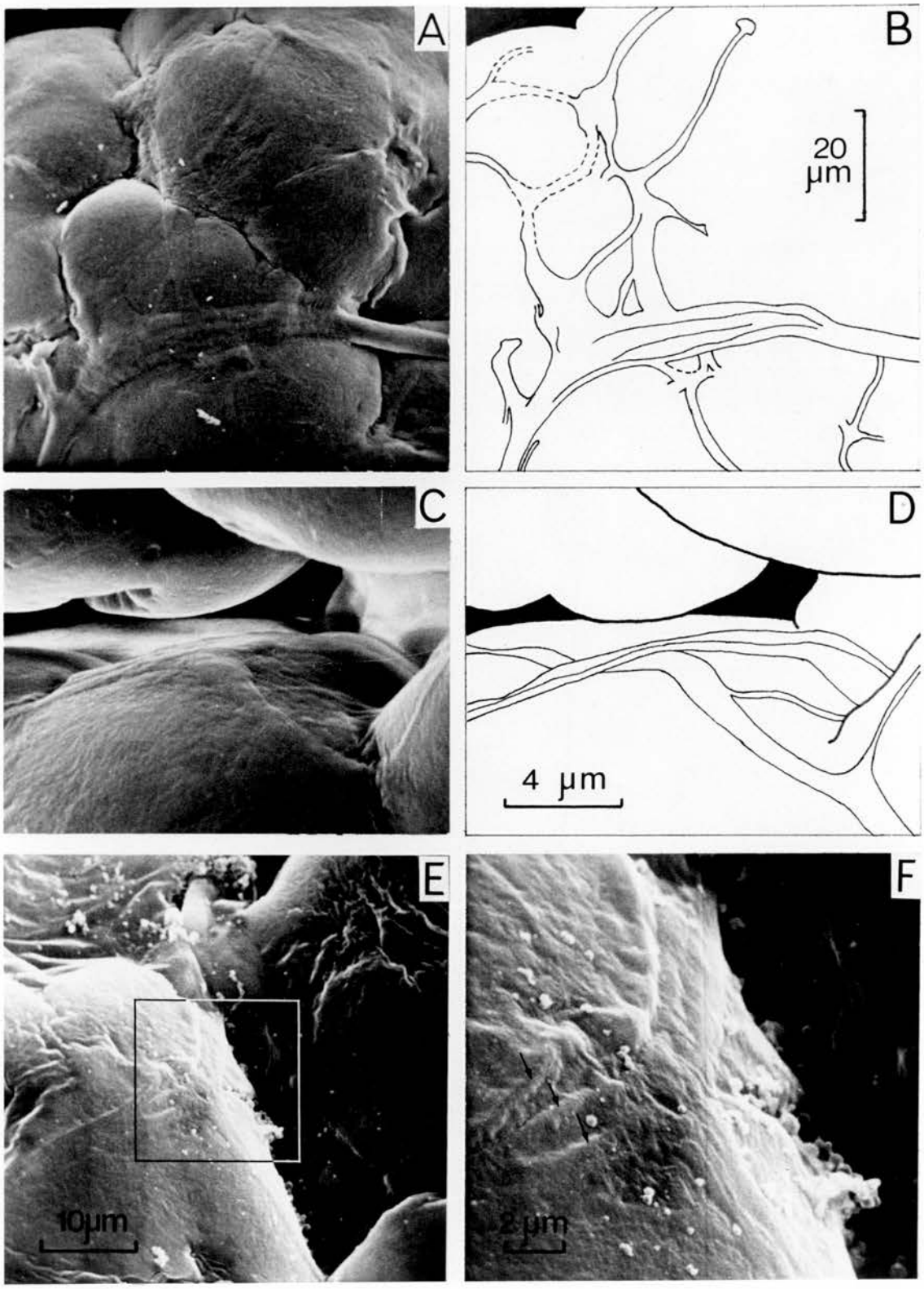
B. Tracing of micrograph in figure A., showing course of acinar axons, recourse being made to other micrographs from different angles and magnifications to confirm their presence. Note nerve trunk passing on to acinus showing multiple axons with complex ramifications on acinar surface.

C. Crossing over of acinar axons on surface.

D. Tracing of figure C, obtained in the same manner as that in figure B. Note upper axon can be seen to be composed of at least two units.

E. Complex junction between three groups of axons on acinar surface.

F. Detail of area outlined in figure E. Note multiple axons (arrows) are associated with the acinar nerves in this area.


A
B
20
µm
C
D
4 µm
E
10µm
F
2 µm

(figs 12; c,d). Investigation at higher magnifications (figs 12; e.f) suggested that this beading may be due to either axonal convulutions or the presence of axonal swellings similar to those observed in the methylene blue stained axons. To resolve this point it was decided to attempt to strip the basement membrane off the acinar surface by an HCl-collagenase digestion method used successfully by Evan et al. (1976) on the kidney tubules and autonomic ganglia of the rat. Application of this rigorous technique to the salivary gland disrupted the structure of many of the glands considerably making it difficult to follow branches of the duct nerve to the acini. In some cases fragments of the basement membrane were removed completely revealing the underlying acinar cells (fig 13b). It was possible to reduce the disruption and fragmentation considerably by varying the parameters of the treatment and supporting the intact glands on a Sylgard resin filled cup throughout.

Glands treated in this way maintained their integrity rather well (fig 13a) and evidence of considerable digestion of the basement membrane could be seen by the presence of debris on the scanned surface not observed in control preparations. Moreover, the outlines of acinar axons could be observed more clearly (figs 13 & 14). As a result it was possible to examine the acinar surface at higher magnifications and trace fine axons of less than 1 $\mu$m (surface dimensions) which apparently terminate on the surface or pass into the acinus (fig 14b). Furthermore,

Figure 12

Scanning electron micrographs of normal salivary glands.

A. Acinus lying on the edge of acinar lobe with simple and complex neural junctions with three adjacent acini. Details of the junctions (marked B & C) are shown in the following figures. In addition the surface axons of this acinus appeared to exhibit swellings (D) and the details of these are also shown in the following figures.

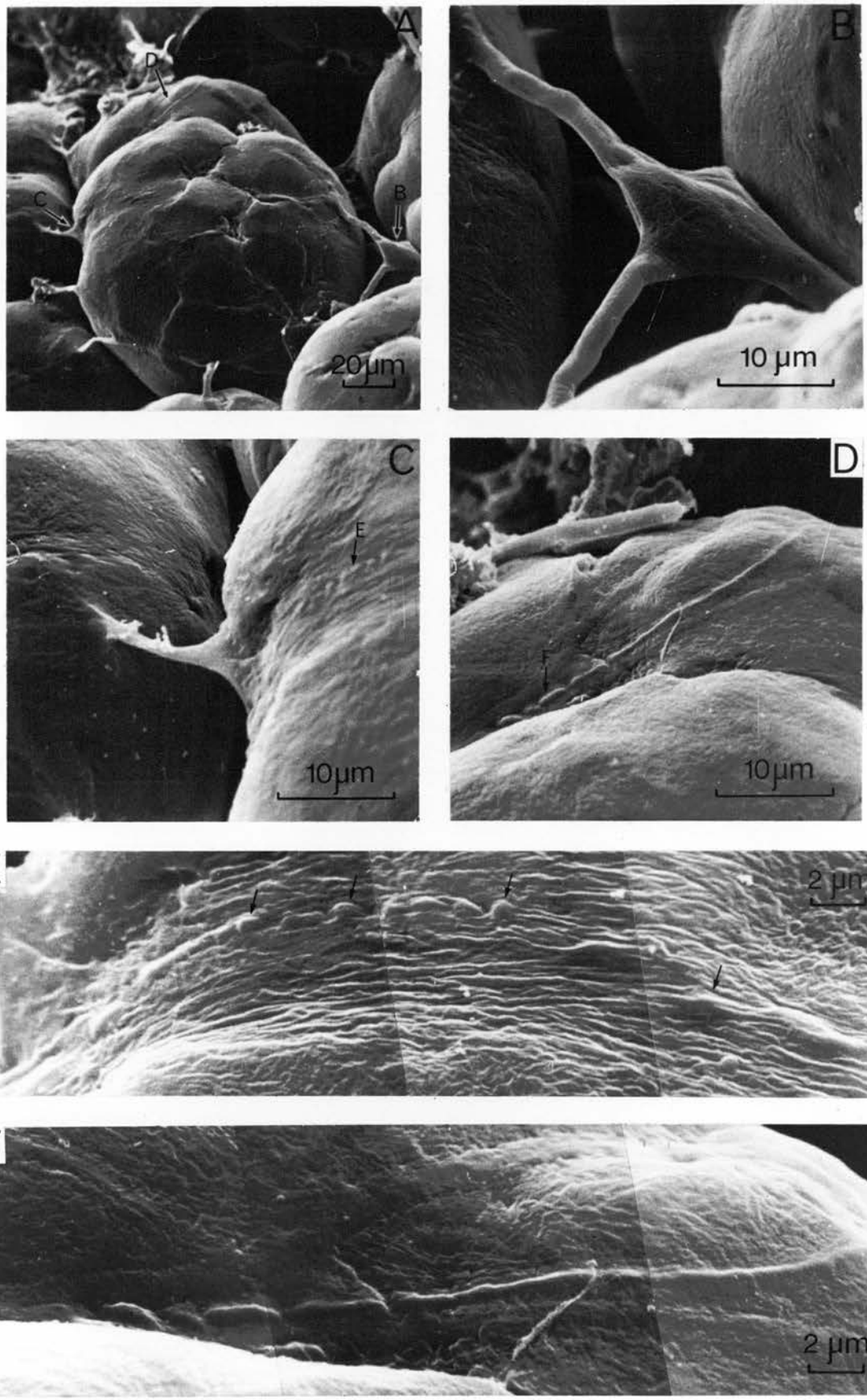
B. Detail of complex junction between at least three acini.

C. Detail of simple junction between adjacent acini. Note also axonal swellings (E).

D. Detail of area marked D in figure A with what appear as either axonal convolutions or swellings marked by arrow at F.

E. Details of axonal swellings (arrows) seen in figure C.

F. Detail of area in figure D. Note complex pattern and convoluted appearance of acinar axons.


A
D
C
B
20µm
B
10 µm
C
E
10µm
D
F
10µm
E
2 µm
F
2 µm

the beaded appearance of acinar axons seen in unstripped preparations was revealed as due to swellings, (figs 13; c,d; 14a) rather than axonal convolutions.

Figure 13

Scanning electron micrographs of salivary glands treated with HCl/collagenase.

A. Low power view of area of treated gland. Note the clarity of the acinar innervation with a branch of the duct nerve (n) passing on to acinar group from the reservoir duct (D).

B. Part of an acinus from a preparation which suffered severe erosion of basement membrane (BM) following prolonged treatment with collagenase (>4 h) where fragments of the basement membrane were completely removed to reveal the underlying cells.

C. Axonal swellings of acinar axon traversing the acinar surface.

D. Same as C in a different preparation.

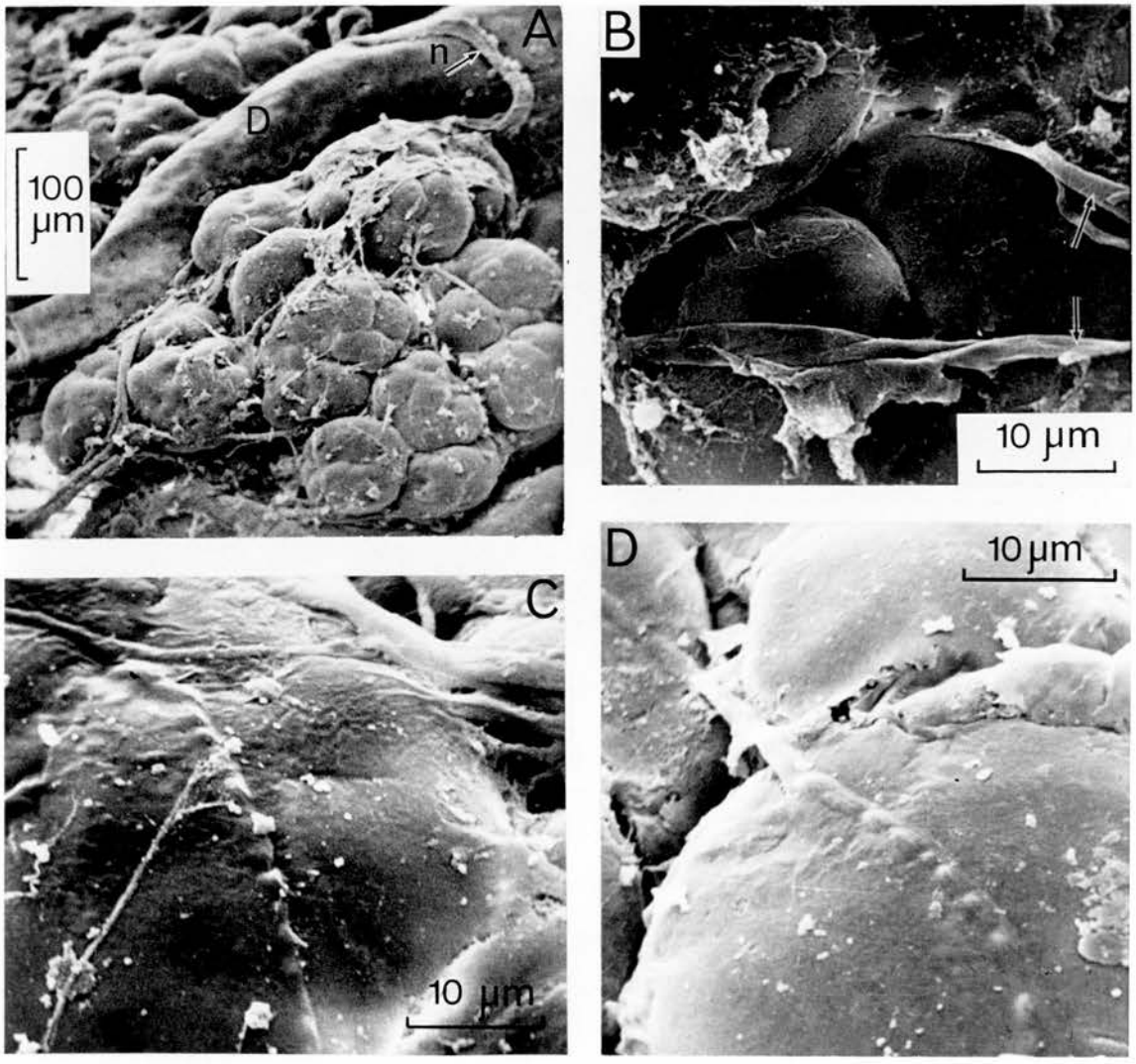
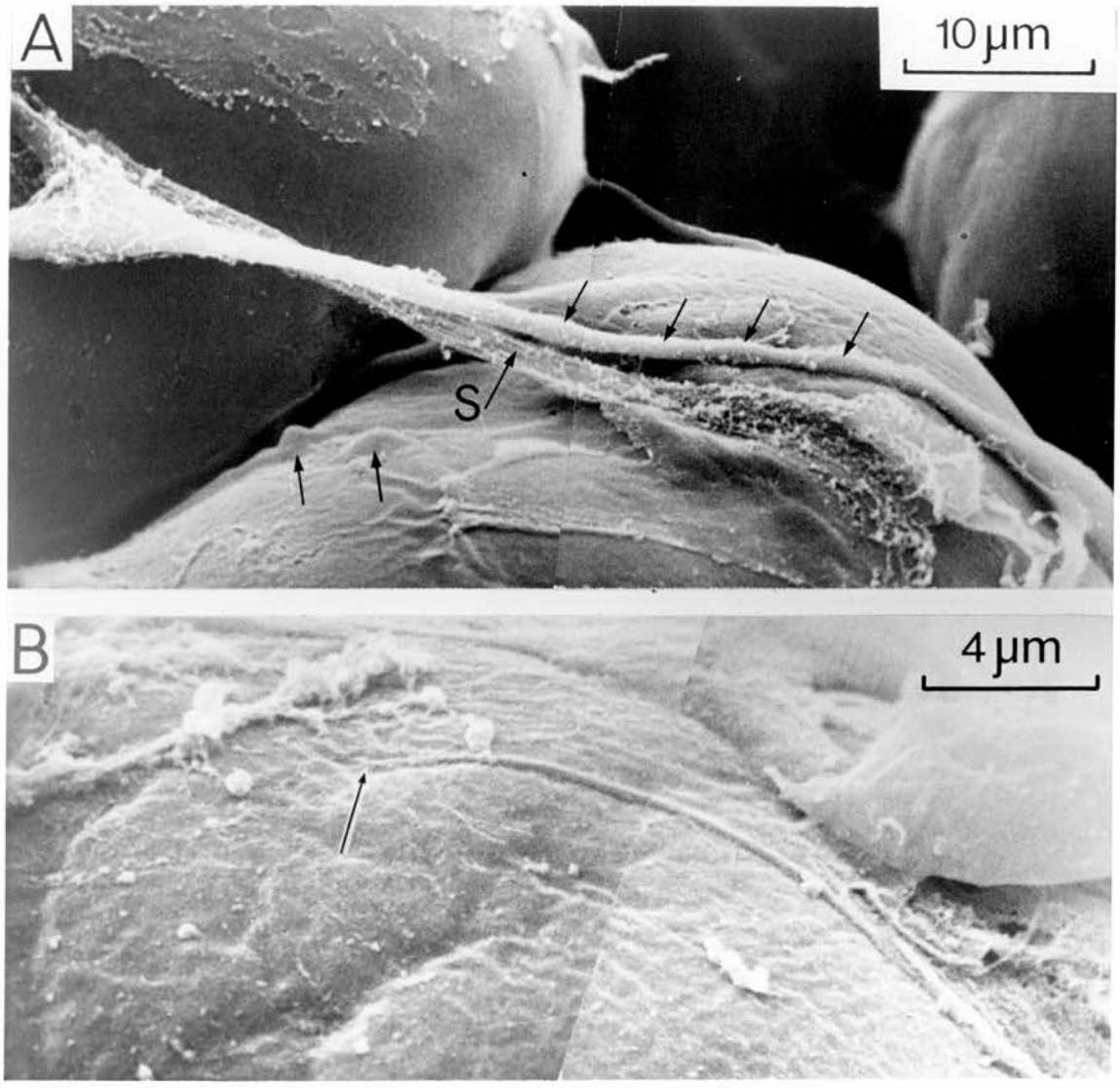

A
n
D
100
µm
B
10 µm
C
10 µm
D
10 µm

Figure 14

Scanning electron micrographs of salivary glands treated with HCl/collagenase.

A. Axon passing between adjacent acini. Note axonal swellings and the remains of the axonal sheath (S). Axonal swellings (arrows) are also evident in another surface axon.

B. Fine axon appearing to terminate (arrow) on the acinar surface.


A
10 µm
S
B
4 µm

Intracellular staining.

To obtain a more complete picture of the innervation of the cockroach salivary gland it was decided to attempt to locate the position of the cell bodies within the CNS of the salivary duct nerves by use of a modification of the axonal filling method of Iles and Mulloney (1971). In addition the same method was employed to investigate the distribution of these nerves within the gland. The intra-axonal markers employed were cobalt chloride and horseradish peroxidase, applied via a suction device (see Methods), no current being used to drive these markers along the axons. The results obtained using this variation in other invertebrates are far superior to those obtained using an imposed current bias (Kater, Nicholson & Davies, 1973).

In this way either one or both of the larger axons of the SDN, found previously with methylene blue, were successfully filled in both directions with HRP or cobalt. However, the presence of cobalt or HRP was not detected in the smaller axons of the SDN found in methylene blue stained glands and with transmission electron microscopy (House, 1977). Nor was it possible to fill the finer branches of the stomatogastric nerve with cobalt even though this marker penetrated down the axon of the main trunk, in an anteriograde direction, to the ingluvial ganglion during exposure periods of 2 - 8 hr.

The chance of filling such axons in mixed fibres is probably low since the lower limit achieved by other

workers (e.g. Sandeman and Okajima, 1973) appears to be in excess of 2 $\mu$m in diameter. Indeed most success with this method has been achieved when filling axons with diameters greater than 10 $\mu$m (e.g. Iles and Mulloney, 1971; Pitman, Cohen and Tweedle, 1973; Tyrer and Altman, 1974).

The morphology of the larger axons on the reservoir duct filled with cobalt and HRP were the same as those seen in the previously described methylene blue stained glands. The diameters of the axons lay between 3 - 5 $\mu$m and they followed the reservoir ducts on their main course towards the gland. The best fill was achieved with cobalt (fig 15) which in this case moved a distance of approximately 850 $\mu$m down these axons in a period of eight hours. The axons could clearly be traced on their course down the ducts. Several branches (fig 15; a,b) left the duct presumably to innervate acini. One of these branches (fig 15c) was composed of at least two axons, less than 1 $\mu$m in diameter, and exhibited, at irregular intervals, 2 - 3 $\mu$m swellings similar to those found by other methods (see table 1). Since the cobalt was not seen to penetrate further down these axons no detailed observations on the acinar innervation could be made.

To fill the larger axons of the duct nerve as far as the first branch, a distance of about 200 - 300 $\mu$m, required a minimum filling time of 2h for cobalt and 4h for HRP; in addition HRP appeared to pass into the connective tissue sheath of the nerve over the initial 50 - 100 $\mu$m and obscured the underlying detail. For

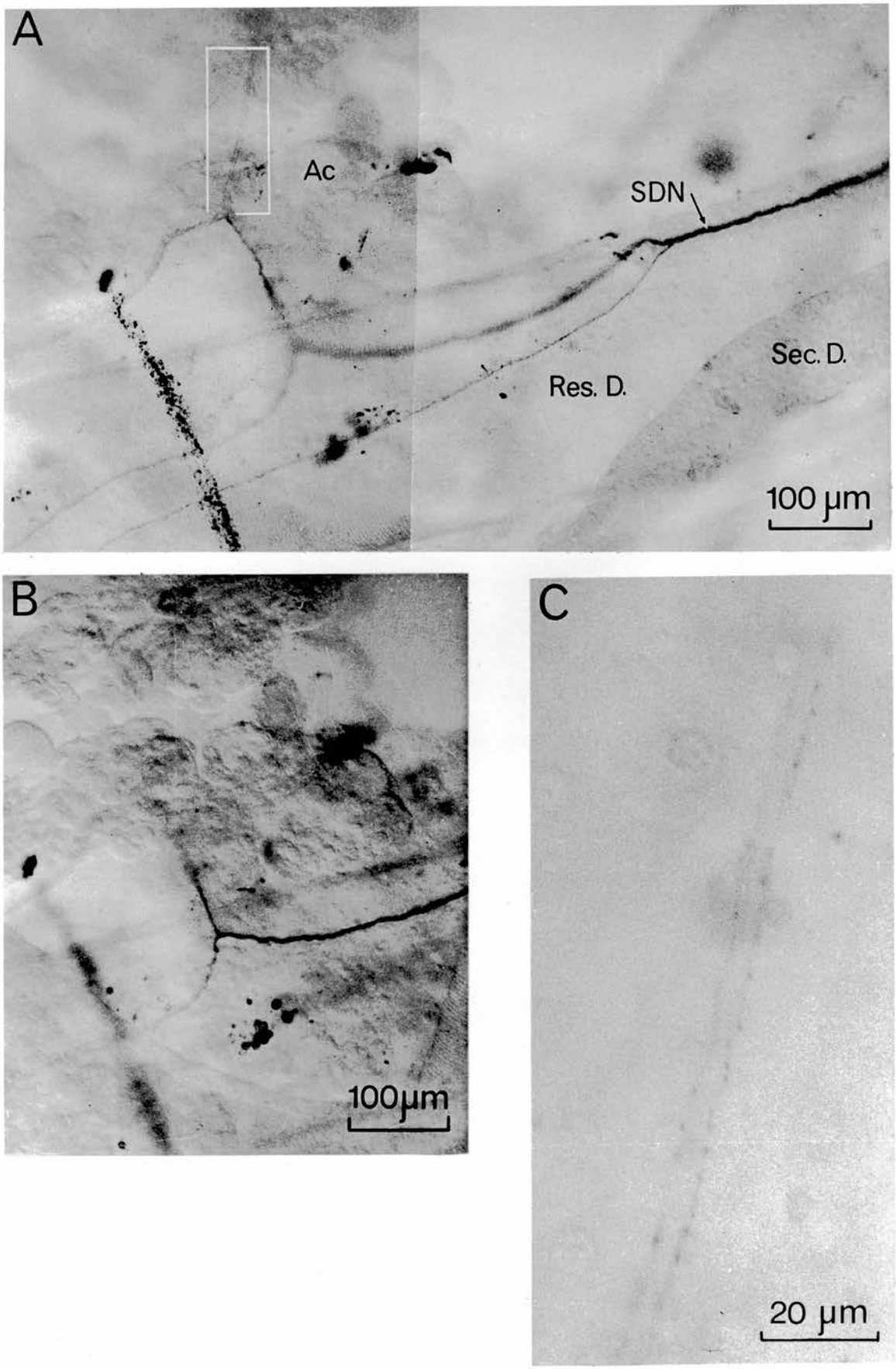
Figure 15

Light micrographs of salivary duct nerves filled with cobalt chloride.

A. Composite showing the course of salivary duct nerve (SDN) down the reservoir duct (Res. D). Note two axons can be clearly seen at the first branch on the duct. Upper branch further subdivides and leaves the duct to form at least four acinar axons.

B. Same field of view as left hand micrograph of composite pair in fig A seen in different focal plane showing branch of SDN leaving duct to form acinar nerves.

C. Detail of acinar axons outlined in fig A. Note presence of irregularly spaced axonal swellings.


A
Ac
SDN
Res. D.
Sec. D.
100 µm
B
100µm
C
20 µm

these reasons the best fills were achieved with cobalt applied for 8 - 10 h; longer periods of exposure produced no increase in the distance moved, probably due to some deterioration of the tissue.

Retrograde filling of the salivary duct nerve was achieved with both HRP and cobalt, revealing neurones with cell bodies within the sub-oesophageal ganglion (fig 16). In each case the cell bodies were ovoid, 40 - 50 $\mu$m long and 25 - 30 $\mu$m wide, with clearly visible nuclei 10 - 15 $\mu$m in diameter. The cells lay approximately 40 $\mu$m below the dorsal surface and slightly lateral to the midline of the ganglion. The axons, 4 - 5 $\mu$m in diameter, initially followed a dorsal course for a short distance and then passed ventrally and posteriorly through the ganglion to exit with the ipsilateral duct nerve on the posterio-apical margin. It is considered that these axons correspond to the larger axons found within the salivary duct nerve with the methylene blue stain.

In eight experiments employing HRP, single cells were found in three ganglia (fig 16a). Two were filled from the left SDN and one from the right. The cell bodies and axons stained dark brown following reaction with DAB, the surrounding nervous tissue remaining unstained. The two cells filled from the left SDN (figs 16a; I,II) were found in a similar position, and lay to the left of the midline and about 50 $\mu$m posterior to the circumoesophageal connective (COC). The cell filled from the right SDN (fig 16a, III) lay to the right of the midline and about

Figure 16

Camera lucida reconstruction from whole mounts showing the position of cell bodies associated with the salivary duct nerve in the sub-oesophageal ganglion. Ganglia are shown dorsal surface uppermost with the exception of fig BII which is seen from its right lateral surface to show the depth of the cell body below the dorsal surface and the general course of the axon seen in the other preparations.

A. HRP filled cells.

I. Cell filled from the left salivary duct nerve; filling time 12 h.

II. Cell filled from the left salivary duct nerve; filling time 6 h.

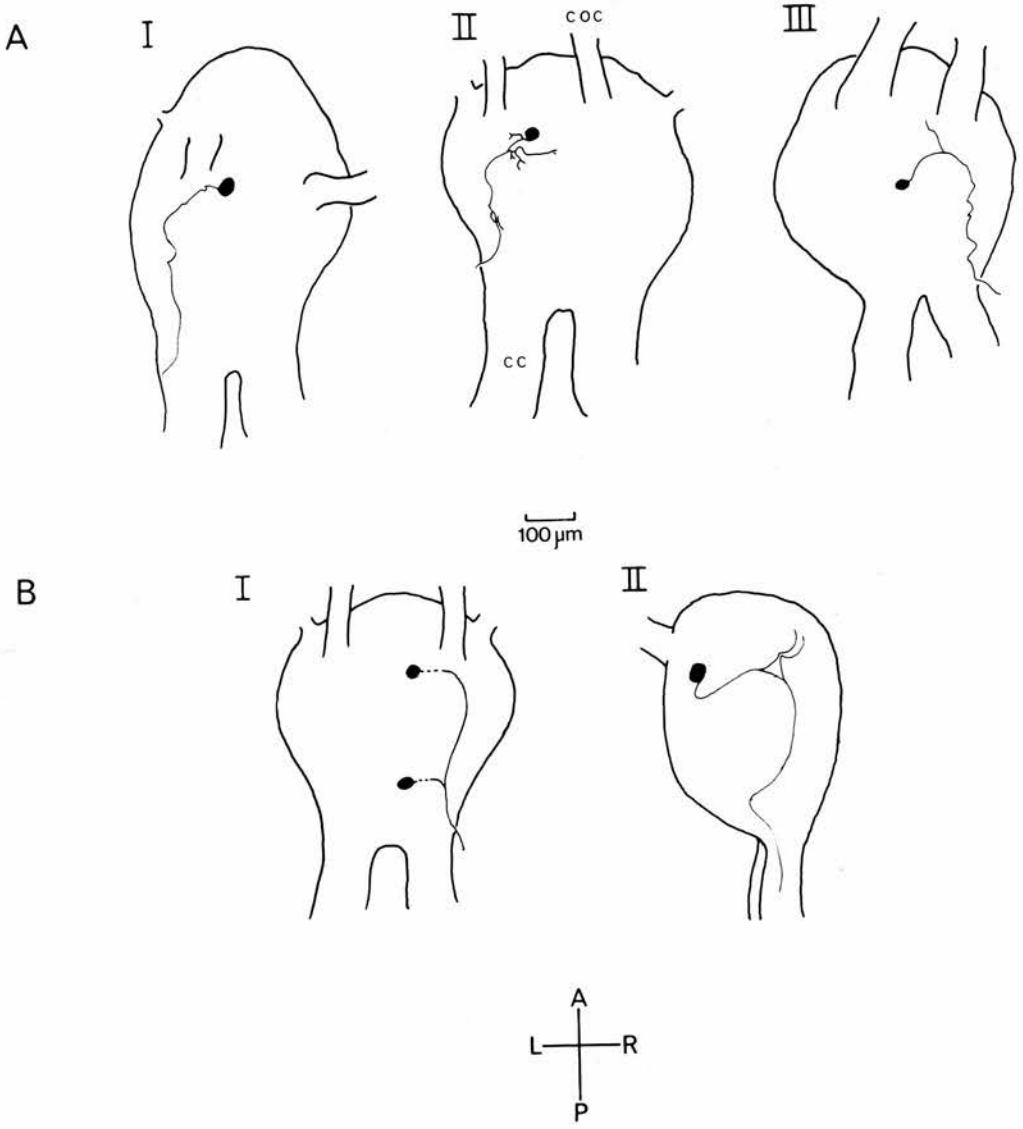
III. Cell filled from the right salivary duct nerve; filling time 6 h.

B. Cobalt chloride filled cells.

I. Cells filled from the right salivary duct nerve; filling time 6 h.

II. Cell filled from the right salivary duct nerve; filling time $4\frac{1}{2}$ h.

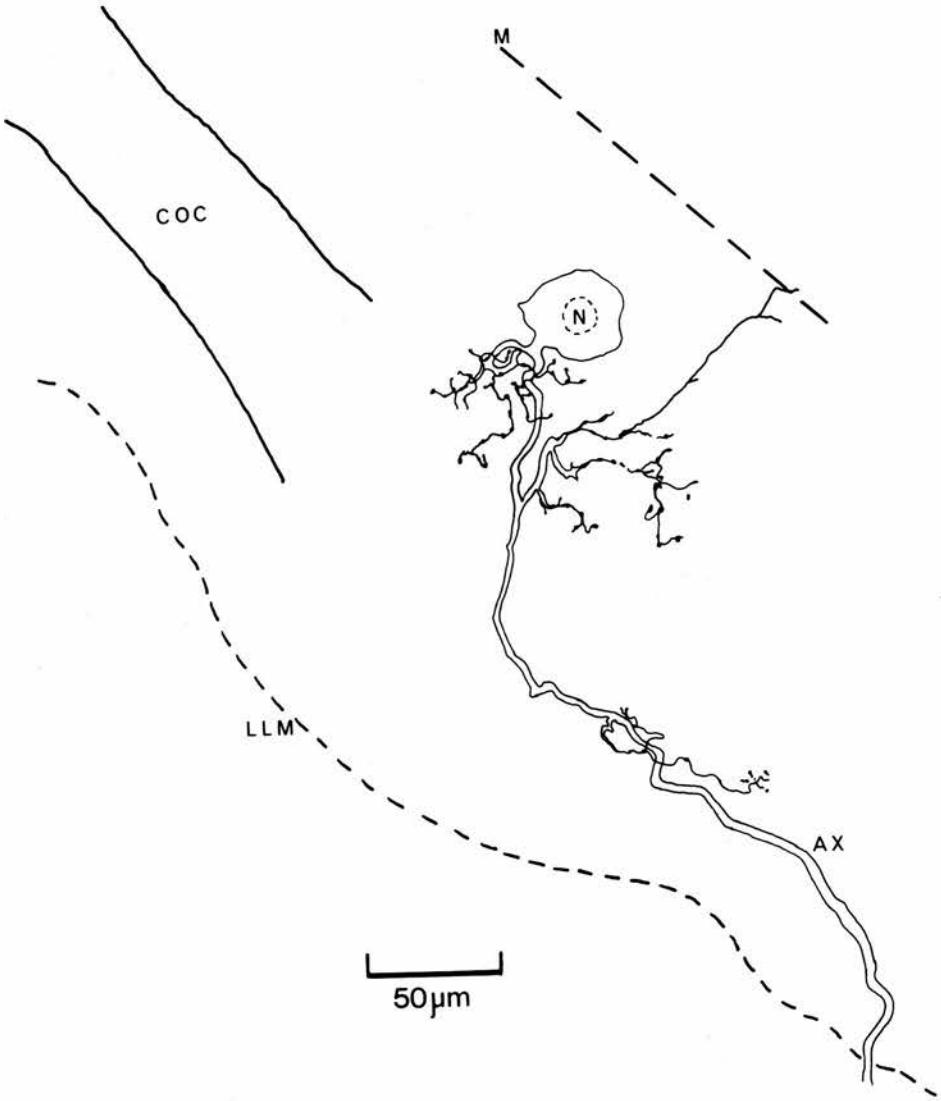
The circumoesophageal connective (COC) was used as a position marker in all preparations.


A
I
II
COC
III
CC
100 µm
B
I
II
A
L
R
P

150 $\mu$m posterior to the COC. Its axon initially followed a more anterior course before giving rise to a short branch 80 $\mu$m from the cell body. This branch passed dorsally before it appeared to terminate at a point which corresponded to the contralateral position of the cell bodies filled from the left. Of the three cells identified with HRP only one showed clear details of its dendritic branches (fig 17). The cell body of this neurone extended laterally into a short neurite which formed a T-shaped junction with the axon. The axon extended in an anterior and posterior direction from this point. The anterior portion spiraled in a latero-dorsal direction giving off fine branched dendrites. The posterior portion of the axon ran in a latero-ventral direction for 50 $\mu$m before it branched medially to form another dendritic tree. The main course of the axon continued in a ventral and posterior direction and gave rise to a branched axon collateral before exiting from the ganglion. The anterior dendritic tree was formed by four primary branches, some of which divided further to give second and third order branches often less than 0·25 $\mu$m in diameter. Some of these fine branches exhibited 1 - 1·5 $\mu$m swellings along their length and at their terminations. The posterior dendritic tree, formed by three primary branches exhibited similar swellings which were less numerous and occurred mainly on or near their terminations. No swellings were observed on the branches of the axon collateral.

Figure 17

Detailed camera lucida reconstruction of HRP filled cell shown in fig 16 AII. Broken lines indicate midline (M) and the left lateral margin (LLM) of the sub-oesophageal ganglion. COC-circumoesophageal connective, N - nucleus.


M
COC
N
LLM
AX
50µm

In the two successful cobalt preparations (10 experiments) the neurones appeared black with the surrounding tissue transparent to pale yellow. In one ganglion, filled from the right SDN, two cell bodies were found (fig 16b, I); both lay to the right of the midline and were separated medially by about 150 $\mu$m. The anterior cell of this pair lay approximately 40 $\mu$m posterior to the COC, this position being similar to the previously described HRP filled cells shown in figures 16a; I,II. The cell in the posterior position corresponded approximately to that of the ipsilateral filled HRP cell in figure 16a, III. The axons of the cobalt filled cells were traced into the SDN and followed the same general course as that described for the HRP filled cells. In the other ganglion, also filled from the right SDN, a single cell was found (fig 16b, II) in a similar position to the anterior cells described above. Whilst no detailed structure of the dendritic trees were seen in any of the cells identified with cobalt, the axon of one cell (fig 16b, II) exhibited branches about 150 $\mu$m from its soma. These branches ran in an anterio-dorsal direction and were not seen to leave the ganglion.

It is clear from the dimensions of the axons of the cell bodies found within the SOG that they correspond to the larger axons found within the SDN. However, these results only account for three of the four larger axons found within the SDN. The position of the cell body associated with the remaining larger axon in the

left SDN was not ascertained, nor was it possible to fill the fine axons in either SDN. The failure to fill this and the smaller axons of the SDN may be due to the sealing over of their cut ends and in addition in the case of the smaller axons the preferential filling of the larger axons within a mixed nerve (cf. Iles and Mulloney, 1971; Kater, Nicholson and Davies, 1973; Sandeman and Okajima, 1973).

Further experiments were performed to ascertain the position of the cell bodies within the CNS. These were based on the evidence presented by Lavail and Lavail (1972) who demonstrated that HRP introduced near the nerve terminals of retinal ganglion cells was transported back to their cell bodies. Salivary glands were immersed in HRP, the SOG isolated in liquid paraffin, for periods of 6, 8, 12 and 24 hrs. Subsequent reaction with DAB stained the acini, secretory ducts and reservoirs dark brown; however no stained cell bodies could be found in the SOG nor was there any evidence of HRP within the salivary duct nerves.

In addition to the experiments described above further attempts were made to identify cell bodies in the SOG by searching for increases in the perinuclear RNA concentration following section of the SDN by the method of Cohen and Jacklet (1965). The SDN was cut unilaterally in 8 cockroaches, the ganglia were removed at intervals from 2 - 5 days and comparisons made between the experimental and control sides. Two normal ganglia

were also inspected. In serial sections of both experimental and control ganglia, treated with pyranine and malachite green, the cytoplasm of the cell bodies stained a uniform pink colour with a darker, thin, perinuclear ring. No increase in the density and width of the perinuclear ring was observed in any of the cells found on the experimental side of the ganglia when compared to those of the controls. Therefore no conclusive results were obtained using this method, as was also the case for locust motoneurones reported by Miller (1967).

# DISCUSSION

Light microscopy discloses a complex innervation of the salivary gland of the cockroach N. cinerea. Because of its open structure it has been possible to use the increased resolution and depth of focus inherent in scanning electron microscopy to study the surface innervation. In addition use of an axonal filling method has revealed the source of part of the innervation within the CNS.

The principle innervation of the gland arises from the sub-oesophageal ganglion of the ventral nerve cord and passes to the gland via the reservoir ducts. A secondary contribution is made by branches of the stomatogastric nerve which is part of the stomadeal nervous system. The salivary glands of the cockroaches, B. orientalis, B. germanica (Hofer, 1887), P. americana (Whitehead, 1971) and the locust, S. gregaria (Klemm, 1972) are innervated in a similar manner; whilst those of the moth, Manduca sexta, are innervated solely by the stomatogastric nerve (Robertson, 1974).

The duct nerves have been traced by light and scanning electron microscopy, branching of these nerves occurring as they pass down the reservoir ducts. The difference in the branching pattern of these nerves when compared to those of P. americana (Whitehead, 1971) may be due to the different topographical relationship between the acinar groups and the reservoirs in each species, described by

Sutherland and Chillseyzn (1968). The number of axons in these nerves can be seen to be as high as eight when investigated by transmission electron microscopy (House, 1977, D.J. Maxwell, personal communication); a similar number has been observed in P. americana (Whitehead, 1971). The nerves are always composed of two large axons, 3 - 5 $\mu$m in diameter and several smaller axons of about 1 $\mu$m in diameter. This composition has been confirmed in methylene blue stained glands and in addition the distribution of the larger axons on the duct has been demonstrated with the axonal filling method.

Retrograde filling of the larger axons with both HRP and cobalt has revealed cell bodies lying within the sub-oesophageal ganglion. Their position within the ganglion corresponds to an area described by Pipa, Cook and Richards (1959) as the dorsal cortex in the sub-oesophageal ganglion of P. americana. This area was shown by Pipa et al. to contain only a few cell bodies and these generally occurred in the medial cleft. The size and shape of cell bodies similar to those found in this study have been classified by these workers as 'giant oviate nerve cells', one of the two major groups of cells found within the cockroach ganglia. Unlike their vertebrate counterparts, and with the exception of sensory receptors, nearly all insect nerve cells have cell bodies that are structurally unipolar; i.e. the perikaryon gives rise to a single process called the cell body fibre or neurite. The neurite links the perikaryon with the

integrative components of the cell and at its entry into the neuropile or tracts it may give rise to a single or branched axon-like fibre. This fibre is either a prolongation of the neurite or is the product of a T or Y shaped birfurcation (see Strausfeld, 1976). The neurone filled with HRP (fig 17) certainly follows this pattern and may be classified according to Strausfeld (1976) as a category Ib multipolar simple neurone, with two dendritic trees and a single axon collateral.

The cell bodies associated with the SDN are clearly divisible into two topographical groups, anterior and posterior, lying on either side of the midline and separated medially by about 100 - 150 $\mu$m. The anterior group appears to be composed of two cells (see fig 16), one on either side of the midline and each cell contributing one axon to the ipsilateral duct nerve. It would not be unreasonable to assume that the posterior group has a similar composition, since the gland has a bilateral symmetrical arrangement and it is clear from their dimensions that the axons from the cell bodies so far identified correspond to the two larger axons found in each SDN. However only one cell in the posterior group, contributing the second large axon to the right SDN, could be found (fig 16), The failure to fill the small axons and the remaining large axon of the left SDN as well as the fine branches of the SGN associated with the gland has been discussed (cf. Iles and Mulloney, 1971; Kater, Nicholson and Davies, 1973; Sandeman and Okajima, 1973). Although

the additional methods of Lavail and Lavail (1972) and Cohen and Jacklet (1965) employed to identify the source of the innervation proved inconclusive the possibility of cell bodies lying within other parts of the CNS has not been excluded.

The tracts of the axons of the cell bodies identified within the SOG follow a remarkably similar course to those described by Hofer (1887) for the salivary duct nerve of B.orientalis. No other work to investigate the position of the cell bodies associated with the salivary gland innervation of cockroaches or other related species appears to have been reported.

That acinar groups can be supplied by more than one branch of the duct nerve, as observed in the present study, is confirmed by the electrophysiological investigations of Ginsborg and House (1976). In their experiments on paired salivary glands, in which the duct nerves on each side were separately stimulated, they found that centrally placed acini received at least one axon from each salivary nerve. Salivary secretion from isolated glands of N. cinerea is elicited following stimulation of the duct nerves (Smith and House, 1977) and in P. americana Whitehead (1971) has observed secretion whilst recording the efferent discharge in the same nerve.

The functional role played by the stomatogastric nerve is unclear. Ginsborg and House (1976) in an electrophysiological study of neuroglandular relationships suggested that the stomatogastric nerve made no conspicuous

contribution and Whitehead (1970) could find no relationship between electrical activity in this nerve and salivary secretion. Nevertheless branches of the stomatogastric nerve, although not so extensive as those of the duct nerves, can be seen to supply the gland in methylene blue stained preparations. Those branches that are associated with acini may only serve to modify or augment salivary secretion rather than initiate it. Evidence to support such a role is tenuous. It relies mainly on the presence of acetylcholinesterase found in the stomatogastric nerve in the present study, and the previous work of Bowser-Riley and House (1976) which suggested a presynaptic transmitter action for acetylcholine on the acinar nerve terminals. However no discrete areas of acetylcholinesterase activity were found in the gland, and attempts to initiate salivary secretion following stimulation of the stomatogastric nerve in intact preparations proved inconclusive (Bowser-Riley and Smith, unpublished observations).

Other branches of the stomatogastric nerve have clearly been observed to join the axons of discrete neurones confined to the anterior regions of the reservoirs. These cells or their ramifying distal processes may act as stretch receptors that could represent some form of sensory system providing information on pressure changes in the reservoir. Such a function was suggested by Osborne (1963) for similar cells in the abdomen of the blowfly larva. Sensory cells of this type have been classified as multipolar type II (sub-cuticular) by Snodgrass (1935)

and are distinct from the unipolar type I (cuticular) cells which are associated with special cuticular sense organs or sensillae. Zawarzin (1916) found numerous sensory cells of type II distributed over the crop of P. americana and traced their axons into the ganglia of the stomadeal nervous system. He observed, in methylene blue stained preparations, that the distal processes of the cells broke up into fine beaded fibres that terminated on the epithelia of the crop. Gelperin (1967) provided electrophysiological evidence for the nature of type II cells on the foregut of the blowfly. He recorded afferent impulses in response to enlargement of the foregut lumen; the impulses were abolished by section of the nerve branch connecting the receptors to the stomadeal nervous system. Both type I and type II cells originate from primary, epidermally derived sense cells, whose axons have grown inwards to join the first peripheral nerve it meets and follow it to the central nervous system (Wigglesworth, 1953). Such a mechanism could possibly account for association of branches of the stomatogastric nerve with the acini; the axons from the type II cells initially joining the acinar axons before passing over to the stomatogastric nerve and continuing on their centripetal course to the CNS. In addition some may follow the duct nerves accounting for some of the finer axons found within this nerve.

Branches of the duct nerve were found to be very extensive with networks of fine axons embracing the acini

to form a plexus. This plexus bears a close resemblance to those described for other insects (Whitehead, 1971; Klemm, 1972) and vertebrates (Hillarp, 1946; Garrett, 1966). This feature of the innervation is apparently analogous to the autonomic plexus reported by Hillarp (1946, 1959) for a variety of mammalian tissues. As the results of this study clearly show, any given acinus is traversed by many axons and this probably indicates multiple innervation of cells within the acinus. This possibility of multiple innervation was first suggested by Lundberg (1955) in the salivary gland of the cat using electrophysiological criteria. Similar evidence has been presented for N. cinerea by House (1973).

Axonal swellings appear as a consistent feature of acinar axons in this and other insect salivary glands when viewed with the light microscope (see table 1 for methods and references). They also occur with similar dimensions in the terminal portions of the autonomic nervous system of mammals when observed using similar methods (Hillarp, 1946; Norberg & Hamberger, 1964; see also Gabella, 1976), and have been described as varicosities by these workers. At the ultrastructural level reconstruction of serial sections of the innervation of the iris dilator muscle in the rat (Hökfelt, 1969) and the superior cervical ganglion of the cat (Elfvin, 1963) demonstrates the presence of axonal swellings and in both cases the presence of large numbers of vesicles within them. In the present study, whilst no statement can be

made about their contents, axonal swellings can definitely be demonstrated by the HCl-collagenase method in conjunction with SEM of axons both on and off the acinar surfaces. The location of these swellings may represent the two types of neuro-effector sites, interstitial and epithelial, postulated by Garrett (1974) for mammalian salivary glands. Swellings under the basement membrane and on the acinar surface (figs 13- c.d) represent the epithelial site and those lying off the acinar surface (but still surrounded by basement membrane) (fig 14a) represent the interstitial site. Ultrastructural studies in this laboratory (D.J. Maxwell, personal communication) are not incompatible with this view.

From the evidence available it is clear that the salivary glands of N. cinerea and certain other insects share some common morphological features with those of vertebrates (including a branching ductal tree; discrete acini invested with a nerve plexus; axonal swellings in the vicinity of the acinar surface). The most obvious difference is the lack of a connective tissue capsule surrounding the invertebrate gland and this has aided the investigation of the cockroach salivary gland considerably.

With regard to the source of the innervation, whilst it is obvious that further experiments are required to complete this aspect of the study, the feasibility of the intra-axonal method was tested and proved successful in locating some of the cells within the CNS. Since their position has been ascertained it should be possible in

future experiments to investigate the morphology of these neurones by intracellular methods. In addition these same methods could reveal the synaptic inputs to these cells and the interactions between them and the activity of the gland.

# SECTION II

# ELECTROPHYSIOLOGY

# INTRODUCTION

The phenomenon of chemically mediated transmission between nerve cells and between a nerve cell and its effector organ has been well documented. A detailed study of this process in complex nervous systems is difficult, and in an attempt to understand some of the basic underlying mechanisms it is useful to resort to simpler systems. Indeed much of our knowledge of transmitter release and its subsequent actions on the postsynaptic effector cell has been elucidated by Katz and his co-workers for ACh acting at the frog neuromuscular junction (see Katz, 1969). Similar insight into the action of NA has been obtained in some peripheral adrenergic synapses (see Haefely, 1972; Bülbring, 1973) and for gamma-aminobutyric acid (GABA) in the inhibitory neuromuscular junction of crustaceans (see Gerschenfeld, 1973; Kuffler & Nicholls, 1976). Whilst the principle of chemical transmission at these synapses amply fulfils the criteria laid down for the identification of such synapses (cf Paton, 1958) there exists a conspicuous gap in our knowledge of such processes. This deficiency lies in the unequivocal demonstration that at a given presynaptic nerve terminal, upon arrival of the action potential, the given transmitter is released in adequate amounts to produce the observed physiological effect. This evidence is so difficult to obtain that it has not been possible to provide it for any transmitter, even

for ACh at the neuromuscular junction.

In contrast to these peripheral synapses little direct information exists about the identity of the transmitters at the majority of central synapses within the vertebrate brain. Nevertheless, for practical purposes, it often seems more plausible to accept the mere presence and appropriate postsynaptic effects of compounds found within neurones as adequate evidence for their presumed transmitter action.

Within this group of compounds there is considerable evidence that dopamine is a transmitter in the vertebrate CNS (see Woodruff, 1971; Hornykiewicz, 1973; Vogt, 1973; Iversen, 1975; Krnjevic, 1975; Hornykiewicz, 1977).

This evidence mainly arises from studies of the nigro-striatal pathway, a neuronal pathway from the substantia nigra to the caudate nucleus; similar but less complete evidence is available for dopamine acting as a transmitter in other areas of the mammalian brain (see Krnjević, 1975; Hornykiewicz, 1977). Initial evidence for the regional distribution of dopamine in the brain of both man and other mammals showed that 80% of the total brain dopamine was concentrated in the nigrostriatal complex (Bertler & Rosengren, 1959; Carlson, 1959; Bertler, 1961). Fluorescent microscopic observation using the formaldehyde method of Falck and Hillarp for the direct visualisation of catecholamines and 5HT demonstrated the specific presence of dopamine within the axons and terminals of neurones of the nigrostriatal

pathway (Anden, Carlsson, Dahlstom, Fuxe, Hillarp & Larsson, 1964; see also Hökfelt & Ljungdahl, 1972). A similar distribution of the dopamine synthesizing enzymes, tyrosine hydroxylase and L-DOPA decarboxylase has also been demonstrated (Côté & Fahn, 1969; Lloyd & Hornykiewicz, 1970; see also Blaschko, 1973).

Following stimulation of the substantia nigra, the release of dopamine or its metabolites (principally homovanillic acid) from the caudate nucleus of the cat has been demonstrated by Portig & Vogt (1969) and increased amounts of dopamine are released within the striatal region (McLennan, 1965; Riddel & Szerb, 1971). Electrical stimulation of the substantia nigra has been shown to elicit unit responses recorded extracellularly from caudate neurones, which were mimicked by iontophoretic application of dopamine to the same cells (McLennan & York, 1967; Feltz & MacKenzie, 1969; Connor, 1970; York, 1970). Although the specific presence of dopamine receptors mediating these responses has not been clearly demonstrated, the actions mediated by dopamine do not appear to involve classical adrenergic receptors (cf Alquist, 1948) since $\alpha$ and $\beta$ blocking agents were not particularly effective in antagonising the pharmacological and electrophysiological responses in the caudate (Connor, 1970, 1972). As far as the caudate is concerned the predominant effect of dopamine appears to be inhibitory, as in the case for other areas of the CNS (Krnjević, 1974, 1975). Possible mechanisms of the

inhibitory action by dopamine have been explored by Krnjevic (1975), who suggested that the inhibitory action may be mediated by a membrane hyperpolarization due to either an increase in potassium conductance or a reduction in sodium conductance. However since no systematic intracellular studies of the action of dopamine on vertebrate central neurones appear to have been performed these views must remain speculative.

It is therefore of interest to have simple preparations on which dopaminergic transmission can be studied in a more direct manner. Several dopamine preparations have recently been explored including the dog renal artery (see Goldberg, Volkman &Kohli, 1978), the intestine of the guinea-pig (Hirst & Silinsky, 1975), mammalian sympathetic ganglia (Libet & Tosaka, 1970) and certain ganglion cells in the mollusc (Woodruff & Walker, 1969; Ascher, 1972; Berry & Cott rell, 1975). The salivary gland of the cockroach N. cinerea may also prove to be such a preparation.

It is evident from the anatomical description given in Section I that the salivary gland of the cockroach presents some distinct advantages for electrophysiological investigation of neuroglandular transmission. The gland is not surrounded by a capsule, so that the acini are accessible for microelectrode recording. It has no blood vessels, smooth muscle or myoepithelial cells; the reservoirs contain a few muscle fibres but their movement is small and generally not troublesome since

the reservoirs can be stretched and used to anchor the preparation. In addition, like many other invertebrate preparations, it is possible to set up *in vitro* preparations and thus control the environment of the tissue.

Intracellular recordings from the acinar cells of the cockroach salivary gland reveals that a hyperpolarizing response can be evoked by electrical stimulation of the salivary duct nerve (House, 1973, 1975). This response evidently arises from an increase in the membrane potassium permeability (Ginsborg, House & Silinsky, 1974), although the participation of other permeability changes has not been excluded. It was noted in previous experiments (House, 1973; House, Ginsborg & Silinsky, 1973) that certain biogenic amines, namely 5HT, adrenaline, noradrenaline and dopamine, also cause hyperpolarization. The hyperpolarization evoked by dopamine is also associated with an increase in membrane potassium conductance (Ginsborg et al. 1974). Salivary secretion from isolated salivary glands of *N. cinerea* elicited by stimulation of the duct nerves (Smith & House, 1977) is also mimicked by bath application of these compounds (Smith, 1977; House & Smith, 1978).

These results are of interest because the presence of catecholamines, but not 5HT, has been demonstrated in the salivary nerve terminals by microspectrofluorimetry (Bland, House, Ginsborg & Laszlo, 1973). Furthermore a radiochemical assay for catecholamines confirmed the presence of dopamine but not noradrenaline in whole gland

extracts (Fry, House & Sharman, 1974).

The evidence against a direct transmitter role for ACh, GABA, glutamate, glycine and alanine in the cockroach salivary gland is unequivocal since none of them produces an electrical or secretory response (Bowser-Riley & House, 1976; House & Smith, 1978). However ACh and the nicotinic agonist carbachol appeared able to influence transmission in the gland as judged by their actions on the neurally evoked response (Bowser-Riley & House, 1976). The results of these experiments form part of this study.

Although the results so far outlined strongly suggest that dopamine could be the transmitter in the cockroach salivary gland the evidence is by no means conclusive. It therefore follows that a similar degree of uncertainty must be assigned to the assumption that the receptors mediating the hyperpolarizing response are specific dopamine receptors.

However indirect evidence on the nature of the receptors can be obtained by use of selective agonists and antagonists to classify the actions of the catecholamines in terms of the receptors involved (see Jenkinson, 1973). This approach is based on the assumption that similarities or differences in the characteristics of responses evoked by these agents reflect similarities or differences in the characteristics of the receptors mediating the response (Furchgott, 1972).

Thus the electrical responses evoked by nerve stimulation or bath applied catecholamines do not appear to

be mediated by classical $\alpha$ and $\beta$ adrenergic receptors since no hyperpolarizations were produced by the selective $\alpha$-agonists amidephrine and methoxamine and the $\beta$-agonist salbutamol (House et. al., 1973; Ginsborg, House & Silinsky, 1976). The classical $\beta$-agonist isoprenaline, which is generally active on such receptors at nanomolar concentrations (Jenkinson, 1973), was found to be inactive at concentrations up to 100 $\mu$M, and at a concentration of 1 mM it was found to be less effective than 1 $\mu$M dopamine (Ginsborg et. al., 1976). The response to nerve stimulation was unaffected by the $\beta$-antagonist propranolol at concentrations up to 20 $\mu$M. However the neurally evoked response was reduced by the $\alpha$-antagonist phentolamine at about 10 $\mu$M, which also reduced a matching response to dopamine by about the same extent, whereas a response to 5-HT remained unaffected (Bland et. al., 1973; House et. al., 1973; Ginsborg et al., 1976).

The action of phentolamine appeared to be reversible and competetive, since the original responses in the absence of phentolamine could be reproduced in its presence by either an increase in the number of stimuli applied to the nerve or an increase in the concentration of bath applied dopamine, and could be restored following removal of phentolamine from the bath (Bland et. al., 1973; House et. al., 1973; Ginsborg et. al., 1976).

The results of the experiments with phentolamine suggested that a more quantitative method of receptor

classification could be employed whereby the affinity constant for the combination between the receptor and the antagonist could be established. Such a method, originally suggested by Schild (1947, 1949), assesses the potency of a given antagonist by measuring the dose ratio i.e. the ratio of concentrations of agonist which produce an equal response in the presence and absence of the antagonist. For a given concentration (I) of antagonist the affinity constant can be calculated using the following expression:-

$$x - 1 = K_I(I) \qquad (1)$$

where x is the dose ratio and $K_I$ the affinity constant for the antagonist (Jenkinson, 1973).

Equation (1) is derived when the law of mass action is applied to the situation where the agonist and antagonist compete for reversible binding to a single population of receptors (see Schild, 1949; Rang, 1971; Furchgott, 1972), and thereby avoids any assumptions about the relationship of the observed response and proportion of receptors occupied since there is not necessarily a linear relationship between the number or fraction of receptors occupied and the size of the response produced (see Rang, 1971; Colquhoun, 1973).

If measurements of the affinity constant of a given antagonist yield comparable values for the blockade of each agonist tested, and the antagonist is known to act in a competitive reversible manner it can be assumed that these agonists all act on the same receptor (Arunlakshana &

Schild, 1959).

Therefore a quantitative study of phentolamine's blocking action has been undertaken, to establish whether this antagonist can distinguish between the receptors for the neurotransmitter and dopamine.

A prerequisite to such an approach is the characterization of the responses to the biogenic amines and nervous stimulation since the net result of the suggested competitive reversible antagonism by phentolamine should be expressed as a parallel shift, to the right, of the log dose/stimulus-response curves of the agonist on the log dose axis. Preliminary experiments have shown that such an approach is possible (Bowser-Riley, 1974; Bowser-Riley & House, 1976).

In addition further experiments were performed to attempt to clarify the actions of ACh in the cockroach salivary gland.

## METHODS

Experiments were performed at room temperature on the isolated salivary glands of adult cockroaches, N. cinerea. The complete salivary apparatus, comprising ducts, acini and reservoirs (fig 2) was removed as described in section I, under the same standard physiological (cockroach) saline.

Two ligatures were attached to the salivary reservoirs associated with the paired gland and a third tied to the connective tissue joining the glands anteriorly.

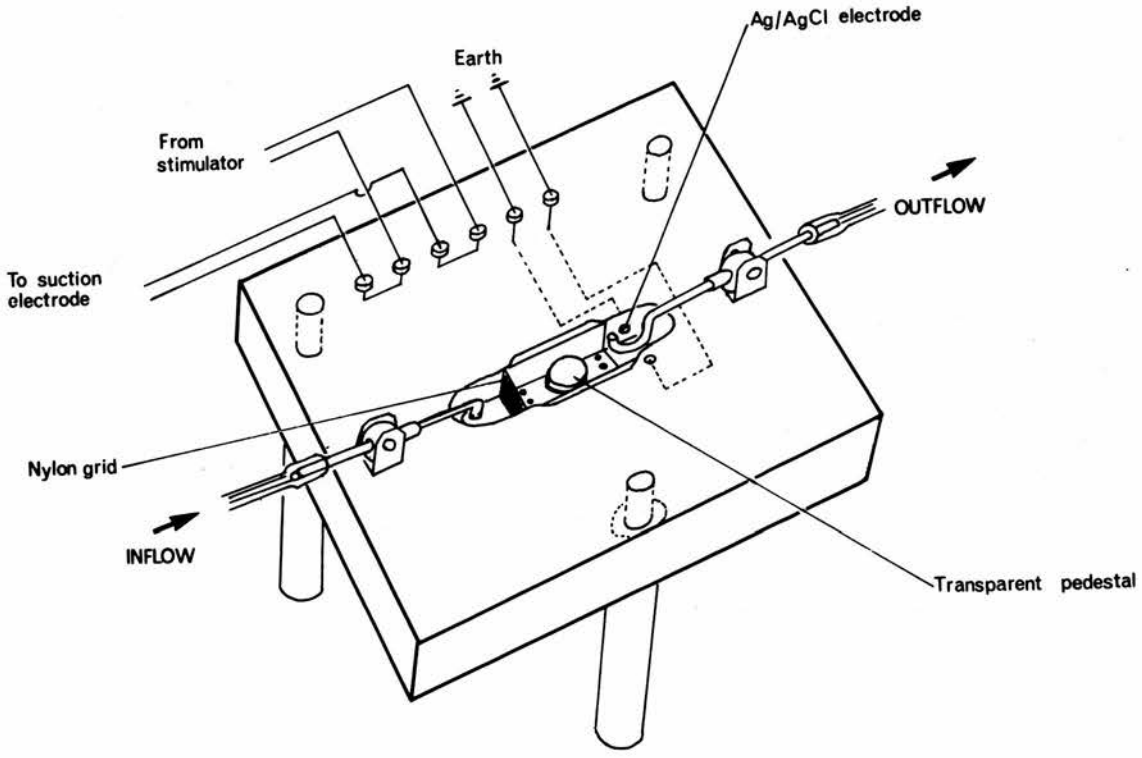
The preparation was mounted in the bath (volume 4·5 ml) shown in figure 18, under the same saline solution as above and stretched over the transparent pedestal by means of the ligatures. The ligatures were anchored by wooden pegs; the tension being sufficient to prevent undue movement of the preparation during perfusion.

The salivary nerves attached to the gland ducts were drawn into a suction stimulating electrode (fig 4A) to lie alongside the anode; the cathode was located in the saline solution surrounding the preparation. The diameter of the glass micro-pipette (fig 4B1) was chosen to give a tight fit around the ducts.

The preparation was routinely perfused with the saline solution at room temperature by using a Watson Marlow H.R. flow inducer set to deliver 2 - 5 ml/minute. Bubbles were excluded by traps which also served to isolate the preparation from any 50 Hz interference from

Figure 18

Diagram of the experimental chamber.


Ag/AgCl electrode
Earth
From stimulator
OUTFLOW
To suction electrode
Nylon grid
INFLOW
Transparent pedestal

the pump motor. Non-turbulent perfusion was achieved by placing a nylon grid between the inflow and outflow of the bath (fig 18).

When test solutions were being applied or washed off, the rate of perfusion was increased to the maximum of 25 ml/min for about 1 minute, so that the bathing solution was rapidly changed. No flow artifacts were observed in the electrical records. The time course of the agonist response was not affected by the perfusion rate provided it exceeded about 7 ml/min. Small bubbles deliberately introduced during changeover of the solutions allowed the arrival at the bath of the new solution to be observed.

The bath was mounted on a damped rigid table to minimize vibration and the preparation observed employing a dissecting microscope (X80), the whole apparatus being enclosed in a Faraday cage.

Membrane potentials were recorded with glass microelectrodes filled with 3M-KCl by the filament method of Thomas (1972); the resistance of these electrodes lay in the range 20 - 40 MΩ. The earth electrodes fixed in the bath (fig 18) comprised solid Ag/AgCl pellets as described by Beranek, Martin & Wicklgren (1970).

The microelectrode was lowered into position by means of a micromanipulator and placed directly adjacent to the acinus. Penetration of the acinus by the microelectrode was achieved by a light tap given to the table on which the apparatus rested. It was assumed that a

microelectrode had entered a cell when, (a) a membrane potential of about -30 mV was recorded, (b) a hyperpolarizing response was elicited by neural stimulation. It has been demonstrated in other experiments with Procion dye-filled electrodes (Bowser-Riley, 1974; House, 1975) that genuine intracellular recordings and neurally evoked responses are obtained from both peripheral and central cells in an acinus.

The microelectrode was connected to the input of a wide-band unity gain Bak electrometer and its potential measured relative to that of the Ag/AgCl earth electrode. The output was fed in parallel to a DC amplifier of a 502 oscilliscope (Tektronix) and an M2 hot pen recorder (Devices) from which permanent records were obtained. Photographic records were obtained with a Nihon Kohden oscillograph camera.

The preparation was stimulated by a square pulse stimulator (Devices) which delivered either single or short trains (2 - 100 at 100 Hz) of pulses (0·5 msec duration, 40 - 60 V amplitude) to the suction electrode. The interval between successive trains was usually 2 - 3 min. The frequency and intervals between stimulus trains were controlled by a Digitimer (Devices).

The effects of the following substances were tested on the membrane potential: acetylcholine chloride, L-adrenaline, carbachol (carbamylcholine chloride), dopamine hydrochloride (3-hydroxy tyramine), 5-hydroxytryptamine creatine sulphate, L-noradrenaline hydrochloride, D.L. octopamine hydrochloride (supplied by

Sigma Chemical Co.); atropine sulphate, physostigmine sulphate (supplied by B.D.H.); phentolamine methylsulphate (supplied by Ciba) and (+)-tubocurarine chloride (supplied by Koch-Light Lab.). Stock solutions of these substances were prepared immediately before use and suitable dilutions were made with the physiological saline given above. The pH of all solutions was checked and appropriate adjustments were made with 1ON-NaOH to give pH 6·9. During the course of the experiments the solutions of the biogenic amines, acetylcholine and carbachol were frequently remade to avoid complications due to their lability at high dilutions.

It was considered that some of these substances might act directly on the gland cell membrane and mimic the electrical response evoked by the actual transmitter. Another possibility is that they might exert indirect effects at the junction by, for example, altering the amount of transmitter released; there are, of course, other possible modes of indirect action. In order to decide if a particular substance acts directly or indirectly not only was the resting potential monitored but also the amplitude of the neurally evoked response.

One of the principal difficulties in studying the actions of these compounds was found to be the maintainance of stable intracellular recording conditions for sufficient periods to complete the experiments. As a consequence of this limitation the undernoted procedures were devised.

Investigation of the stimulus response relationship was as follows. Single stimuli were applied via the suction electrode, to the salivary duct nerve and the ensuing hyperpolarizing response recorded. The amplitude and interval between stimuli were adjusted so that maximal reproducible responses were obtained to single supramaximal stimuli (see House, 1973; Ginsborg & House 1976). If at least four consistent responses were obtained to each of four consecutive stimuli the number of stimuli was incremented in each successive train until a maximal response was obtained. Checks for changes in sensitivity and consistency of responses were obtained by interpolating single stimuli and repeating low, middle and high numbers of stimuli at the end of the procedure. Cells that exhibited marked fluctuations in membrane potential or sizes of response to a given number of stimuli were rejected. Results were expressed as plots of peak values of the response against number of stimuli.

Dose response relationships of the biogenic amines were investigated as follows. At the start of each experiment the maximum response to a train of stimuli (e.g. 50) was obtained for comparison with the maxima evoked by the bath applied agonists. The stimulator was then switched off and a test solution of the given agonist at a concentration of $5 \times 10^{-8}$M was applied by perfusion as described above. The application was maintained until the resulting response had reached a

maximum and then the preparation was rapidly washed with the normal saline, the perfusion rate being reduced when the recorded potential approached its resting value. The test concentration was the same for all agonists and the magnitude of the resulting response served as a reference point for the sensitivity of each agonist. Concentrations above and below this value were then successively applied to the gland until threshold and maximum response values were observed. The interval between successive applications of the agonists was not less than four minutes so that reproducible responses could be obtained (see Bowser-Riley, 1974). Checks for changes in sensitivity were made by repeating doses that gave approximately 30 and 60% of the maximum evoked response. Occasionally responses in certain preparations were slow to recover; these preparations and those that showed marked changes in sensitivity were discarded.

Whenever stable recording conditions were maintained the above procedures were repeated in the presence of phentolamine. When solutions containing phentolamine were applied to the preparation these were perfused initially at 25 ml/min for about 2 minutes to ensure rapid mixing in the bath and thereafter at 2 - 5 ml/min. The inhibition of agonist or neurally evoked responses by phentolamine was not examined until this antagonist had been present in the bath for at least 20 minutes when its blocking action would have reached a maximum (Ginsborg et. al., 1976).

However, since the minimum period of stable recording required to establish a single dose response or stimulus response relationship for any single cell was about 1 h and in the region of 2 - 3 h for the additional experiments in the presence of phentolamine this experimental format for the quantitative study of phentolamine's action was rarely achieved. Indeed, as an indication of the difficulties involved, only in approximately one in five of all cells studied were stable recording conditions in excess of 1 h achieved and of these only about one half gave a stable period in excess of 2 h. Because of this limitation a modified experimental format was devised to study the actions of phentolamine.

In the case of the neurally evoked response consistent maximal responses to single supramaximal stimuli were obtained as described above and cells were selected that gave a response in excess of 20 mV to this stimulus (see later). In these cells stimulus-response relationships were established for 1 - 4 stimuli and maximal responses ascertained when equal responses were obtained to two consecutive large numbers of stimuli. Sensitivity checks were performed as above and the response to the high number (e.g. 8) of stimuli was used to monitor the blocking action of phentolamine. Phentolamine was applied for a period of not less than 20 min before a similar stimulus response relationship was established. If stable recording conditions were maintained additional values were obtained and the phentolamine was washed off

and the recovery observed using the test stimulus.

For agonist-induced responses measurements of the dose-response relationship were confined to concentrations that evoked minimum and maximum responses and to two additional concentrations that gave about 30 and 60% of the maximum response. Sensitivity checks were performed as above and the 60% response used to monitor the blocking action of phentolamine to establish the degree of inhibition and a similar dose response relationship established around this point. If stable recording conditions were maintained the phentolamine was washed off and the recovery monitored using the initial 60% response dose.

The actions of compounds other than phentolamine or the biogenic amines were tested on the resting potential and sub-maximal responses to either nervous stimulation or bath applied dopamine.

# RESULTS

## Neurally Evoked Response.

Previous studies (House, 1973, 1975) on the electrical characteristics of the cockroach salivary gland have reported membrane potentials of about -32 mV following insertion of a microelectrode into an acinar cell. The electrical response of the acinar cells evoked by single supramaximal stimuli applied to the salivary duct nerve comprises a hyperpolarization which varies in amplitude between 1 - 50 mV with a latency of about a second, time to peak amplitude of about 2 seconds and an overall duration of approximately 10 seconds (House, 1973, 1975; Ginsborg & House, 1976). The hyperpolarizing response is occasionally followed by a relatively slower and smaller depolarization. Ginsborg & House (1976) suggested that these opposite phases were independent processes activated by different receptors. Although different transmitters may be involved it is not necessarily so since iontophoretic application of dopamine is also capable of producing a similar biphasic response (Blackman, Ginsborg & House, 1978).

The purpose of the present study was to examine the characteristics of the neurally evoked hyperpolarizing potential to establish whether the method of equal responses (see page 100) could be applied to the inhibition by phentolamine of endogenous transmitter action.

The relationship between stimulus strength and the

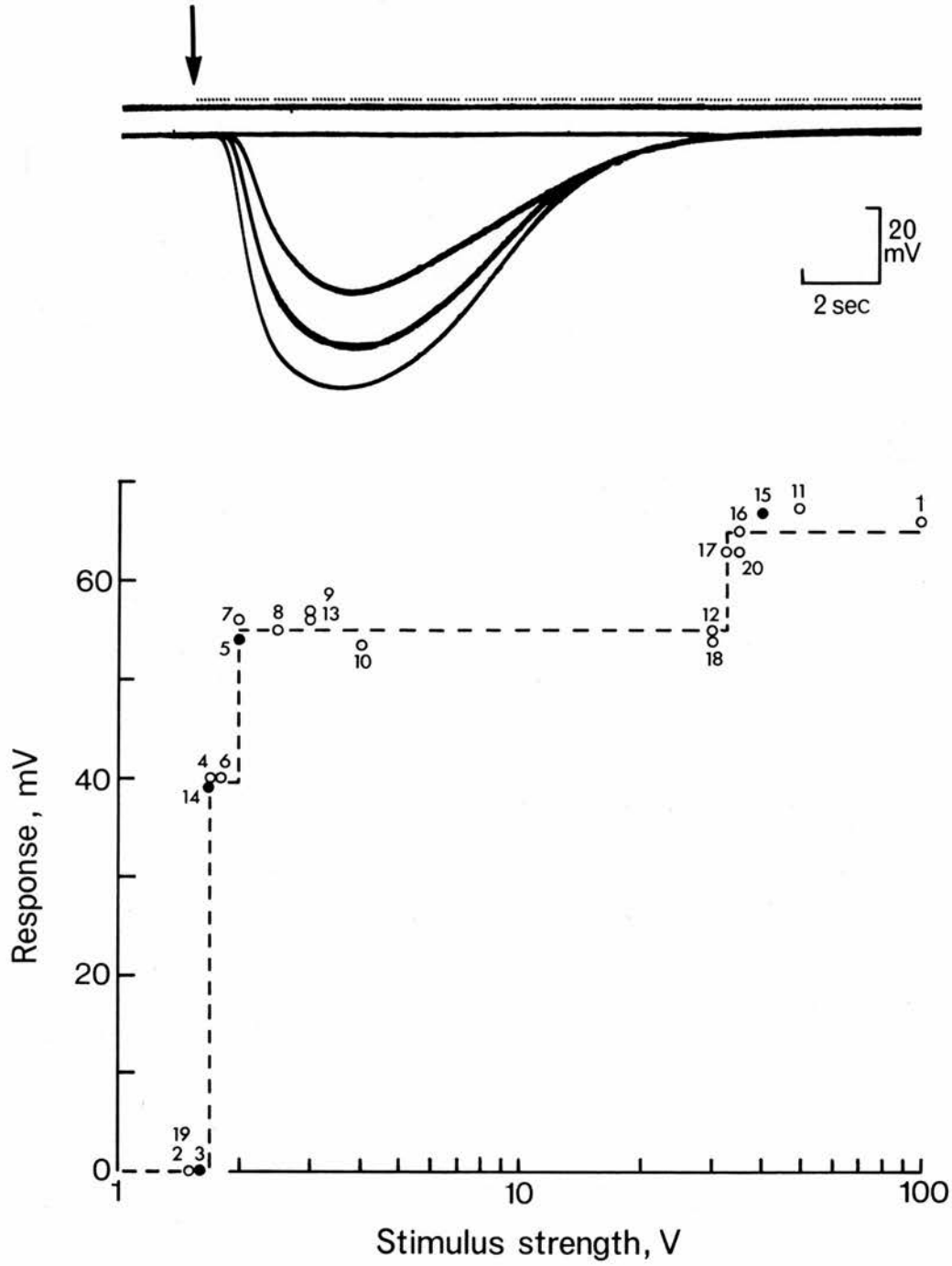
neurally evoked response has been explored by House (1973) and Ginsborg & House (1976). Their results showed that the strength of the stimulus affected the size of the response in a manner suggesting that the acini have multiple innervation.

An example of this relationship is shown in figure 19 where responses were evoked by single stimuli of 0·5 msec duration. Clearly the strength of the stimulus influences the size of the response. In this experiment no response was obtained when the stimulus amplitude was below 1·7 V, but stimuli above that threshold evoked responses which showed some grading with stimulus amplitude. The gradation proceeded in a stepwise fashion with additional thresholds at 2·0 and 32·5 V which indicates that this particular cell was influenced by the activity of at least three axons. In other experiments it was found that there was some variability in the extent to which individual acini were multiply innervated, some acini appearing to be innervated by only one axon whereas others had as many as four (see also Ginsborg & House, 1976). It should be noted that no inferences can be made about the innervation of individual cells since they are electrically coupled and the response is thus related to the activity of all the cells within an acinus (Bland & House, 1971; Ginsborg et.al., 1974; House, 1975).

The main purpose of these experiments was to establish the stimulating conditions for maximum responses evoked

Figure 19

The relation between the stimulus strength and the amplitude of the hyperpolarizing response recorded from a single acinar cell. The peak values of the response to a single stimulus (0·5 msec) has been plotted against the log of the stimulus strength. In this preparation there appear to be thresholds close to 1·7, 2·0 and 32·5 V. The broken line represents the mean value of the response evoked between these thresholds. The number above each point on the graph represents the order in which the stimuli were delivered. Selected responses near to each threshold (filled circles on the graph) are shown above; the arrow indicates the time of stimulation and the upper trace shows the time marks. The resting potential of this cell was -35 mV.


20
mV
2 sec
Response, mV
0
20
40
60
1
10
100
Stimulus strength, V
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

by single stimuli to avoid complications caused by the recruitment of sub-threshold axons following the application of stimulus trains to the salivary duct nerves. The experiments reported in the rest of this study were performed with supramaximal stimuli of 40 - 90 V with a duration of 0·5 msec.

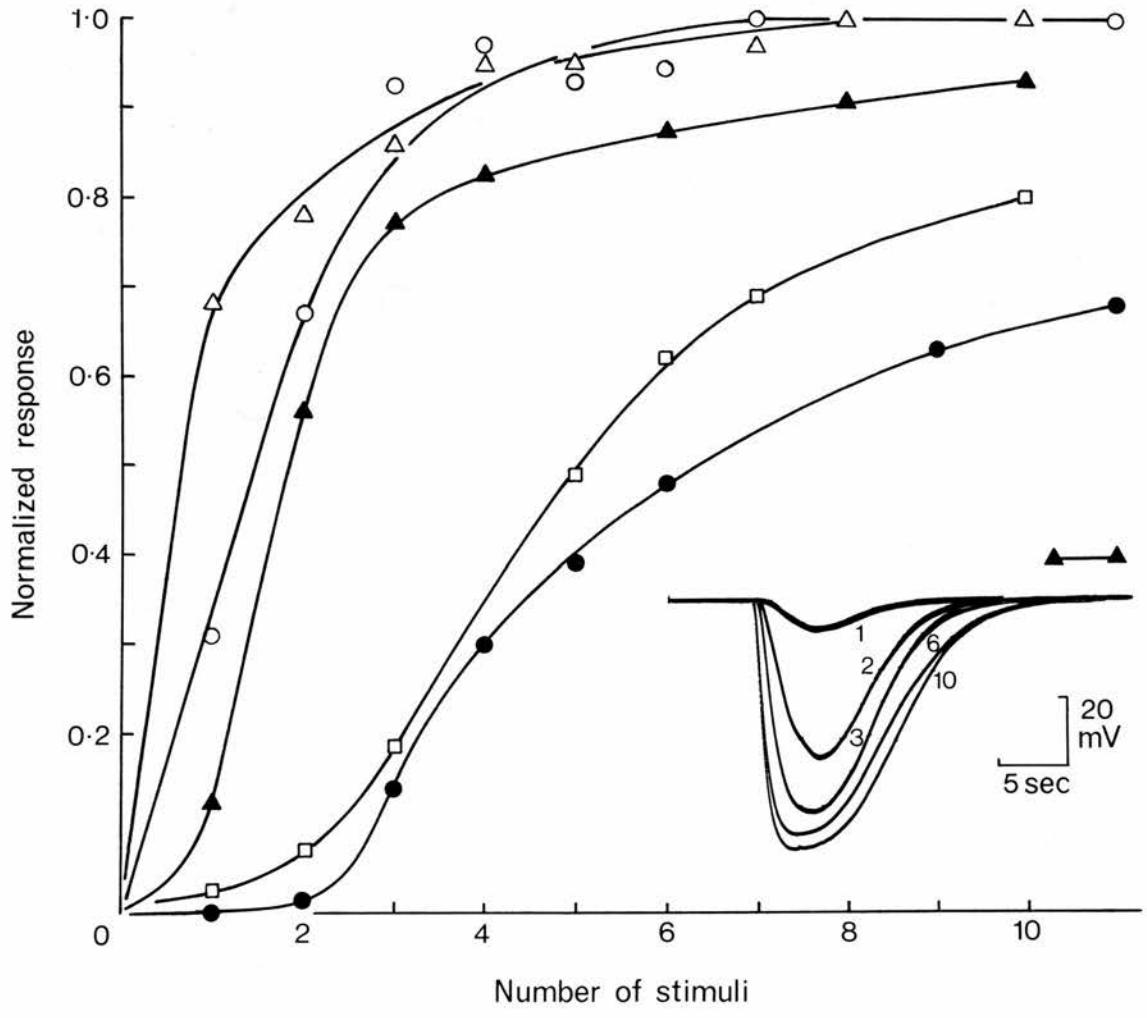
Under these conditions it was found that the amplitude of the hyperpolarizing potential was graded with the number of stimuli applied to the salivary duct nerve. Responses to short trains of stimuli increased the amplitude and duration of the response, maxima were obtained usually with about 30 stimuli and additional stimuli progressively increased the duration of the response. The interval between successive trains required to be greater than two minutes so that reproducible responses could be obtained (see House, 1973; Bowser-Riley, 1974). The relationships between amplitude and number of stimuli are shown in figure 20 for several different cells, and are plotted as peak values of the response against the number of stimuli. The responses have been normalized by dividing them by the corresponding values evoked by large numbers of stimuli. The inset illustrates some of the intracellular recordings from one of them. The stimulus-response relation of this cell had an inflexion near its origin and a similar notable non-linearity is apparent near the origin of some of the other curves (fig 20). This phenomenon has been noted before (Ginsborg & House, 1976) and generally is found in

preparations where a single stimulus produces no response or a relatively small one (< 15 mV). Provided that a single response exceeded about 20 mV, however, there was no non-linearity in the stimulus response relationship (fig 20), the stimulus response curves of these cells being rectangular hyperbolas. In such cases it was found that when the size of the response was plotted against the logarithm of the number of stimuli a dose-response curve was obtained with a similar slope to the corresponding curve for dopamine or noradrenaline obtained in the same cell (see later, and figs 32, 33).

Since the amplitude of the hyperpolarizing response is graded with the number of stimuli, this suggests that the method of equal responses could be applied to investigate the actions of phentolamine on the endogenous transmitter provided this inhibitor exerts no presynaptic actions.

Figure 20

Typical stimulus-response curves for neurally-evoked hyperpolarizing responses in acinar cells. Each symbol denotes a separate experiment on a different preparation. Some representative intracellular recordings of the responses from one of these cells are superimposed in the inset, the number of stimuli being placed beside the corresponding response. The resting potential of this cell was -32 mV and its stimulus-response curve is indicated by ▲. For each cell responses were normalized by dividing them by the corresponding maximal response.


1·0
0·8
0·6
0·4
0·2
0
Normalized response
2
4
6
8
10
Number of stimuli
1
2
3
6
10
20
mV
5 sec

Biogenic amine evoked responses

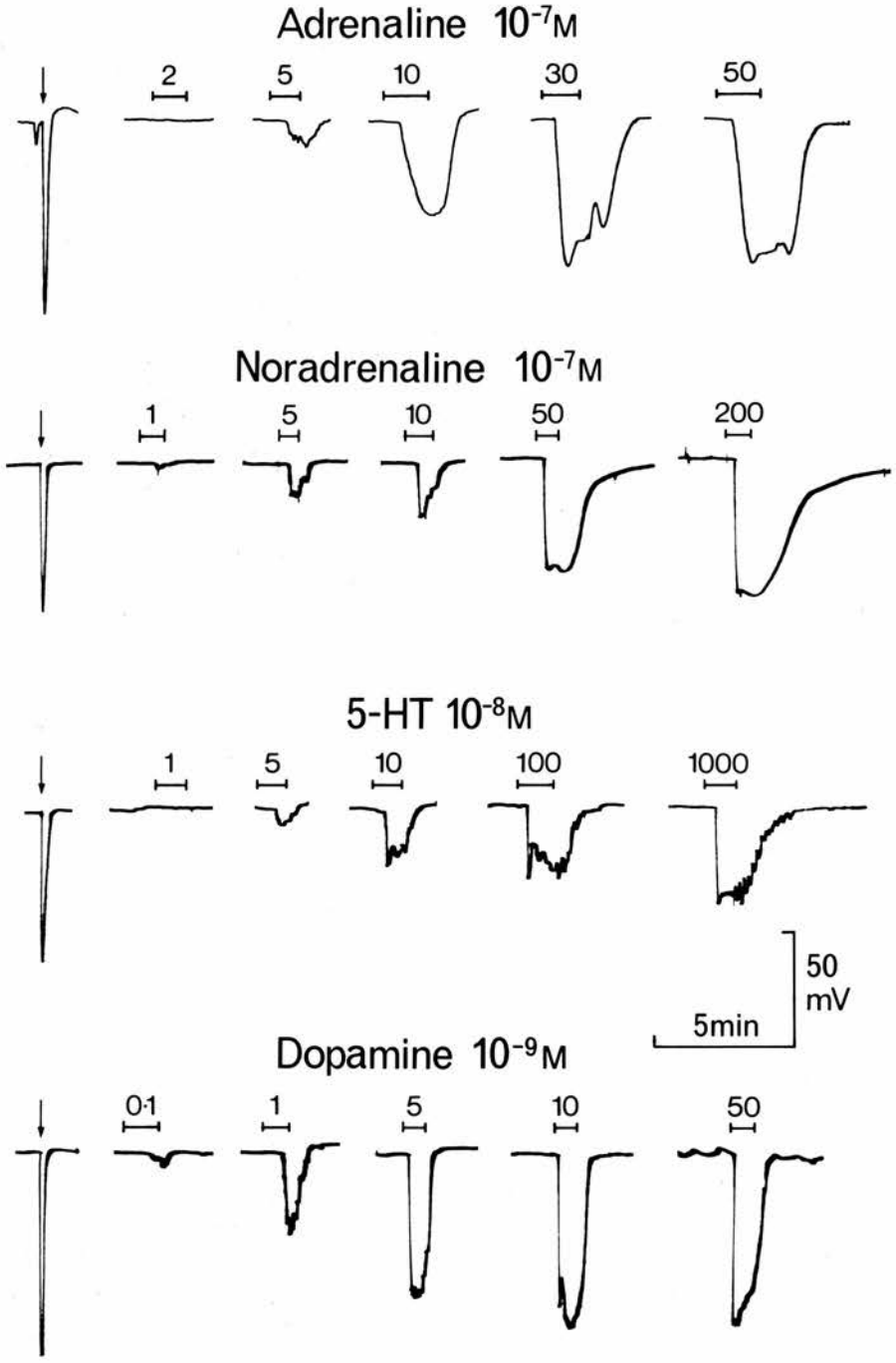
A number of biogenic amines have been shown to hyperpolarize the acinar cells in the cockroach salivary gland (House, 1973; House et al., 1973), and it was decided to investigate their dose-response relationships more fully than previous work had allowed. Each dose-response curve was obtained in a different preparation.

Some typical responses to the biogenic amines adrenaline, noradrenaline, 5-HT and dopamine are shown in figure 21; evidently all these compounds are agonists as judged by their ability to hyperpolarize acinar cells. The maximal response evoked by repetitive nerve stimulation is shown on the left followed by the responses to these agonists applied at different concentrations in the bathing fluid. Clearly the amplitude of each agonist response depends on its concentration and with the exception of 5-HT the maxima attained are the same as that for the neurally evoked response. The time course of the agonist responses is longer than that of the neurally evoked responses, presumably because of their relatively slow application. It has already been demonstrated that the duration of the neurally evoked response can be lengthened to match such agonist responses by maintaining nerve stimulation for a suitable period (Bland et al., 1973).

Dose-response curves for these substances are shown in figure 22, each agonist being studied on a different cell. These particular examples have been selected to

Figure 21

Electrical responses produced by certain agonists. Each set of records for a given agonist was obtained in the same acinar cell and is accompanied (on the left) by the maximal response caused by repetitive nervous stimulation (arrow). Period of bath application is indicated by the horizontal bars and the concentration by the numbers above each response, viz. Adrenaline at $2 \times 10^{-7}$, $5 \times 10^{-7}$, $10^{-6}$, $3 \times 10^{-6}$ and $5 \times 10^{-6}$ M.

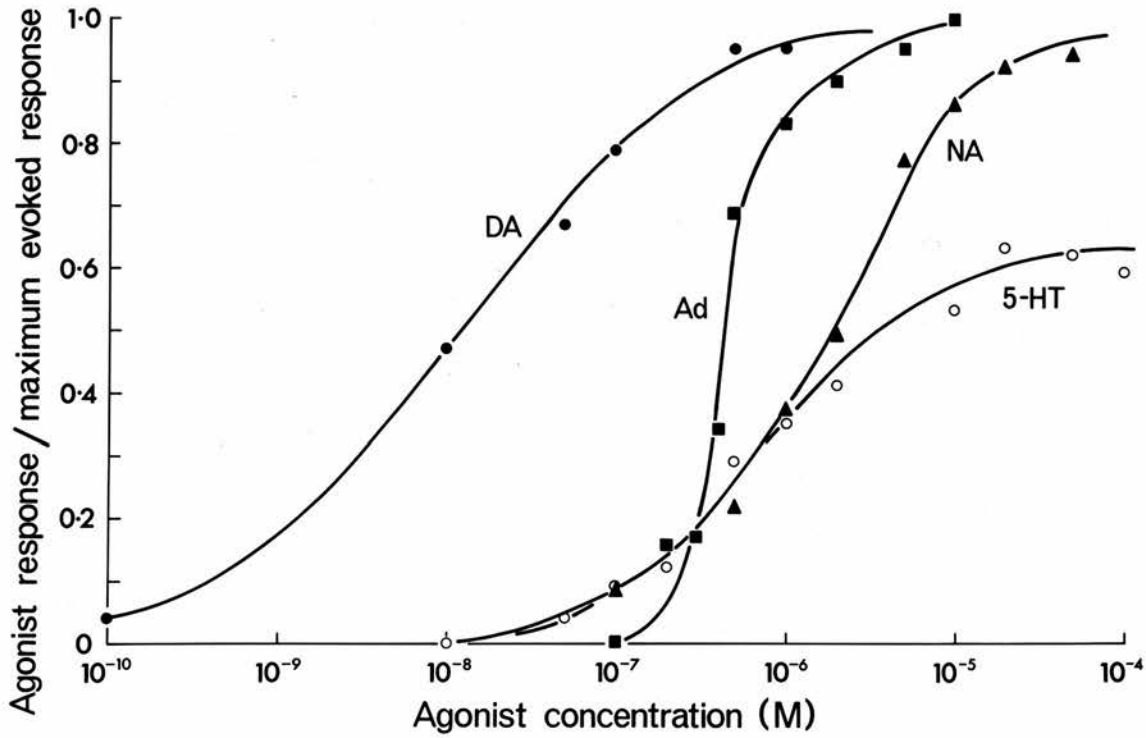

Adrenaline $10^{-7}$M
2
5
10
30
50
Noradrenaline $10^{-7}$M
1
5
10
50
200
5-HT $10^{-8}$M
1
5
10
100
1000
50
mV
5min
Dopamine $10^{-9}$M
0·1
1
5
10
50

emphasize the differences between the curves. To facilitate comparison of results obtained on different cells the agonist responses from each cell have been normalized by dividing their amplitudes by that of the corresponding maximum evoked responses. The values of these maxima are given in the figure legend. It was not possible to obtain curves for all agonists in the same cell as the period of stable recording was invariably too short. However, the dose response curves illustrated in figure 22 reflect the general trends observed in 16 cells with dopamine, 13 with noradrenaline, 10 with adrenaline and 11 with 5-HT. Two of the 16 cells on which dopamine was tested had a high sensitivity to this compound. Some of the responses to one of them are shown in figure 21 and the dose response curve for the other is shown in figure 22. In both cases the threshold concentration for the electrical responses was about $10^{-10}$ M whereas the usual threshold was about $10^{-9}$ M. One of the other cells studied had a threshold of $5 \times 10^{-8}$ M; there appears to be no reasonable explanation for its relatively low sensitivity since the maximum dopamine response attained was similar to that of the more sensitive cells.

All the agonists except 5-HT were able to generate a maximum response equal to the maximum neurally evoked response. Table 2 gives the mean values of the agonist concentrations required to yield a half-maximum response and also the slopes of the linear portions of the log

Figure 22

Typical log. dose-response curves for certain biogenic amines. Each agonist response has been divided by the corresponding maximum neurally evoked response for each cell. These maxima were 76, 65, 58, and 68 mV for dopamine (DA), noradrenaline (NA), adrenaline (Ad) and 5-hydroxytryptamine (5-HT) curves respectively.


Agonist response / maximum evoked response
1·0
0·8
0·6
0·4
0·2
0
DA
Ad
NA
5-HT
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Agonist concentration (M)

dose-response curves. Dopamine is clearly the most potent agonist, noradrenaline and adrenaline are almost equipotent, and 5-HT is apparently only a partial agonist since it failed to produce a maximum equal to that of the evoked response.

TABLE 2

Parameters of dose-response curves for different agonists.

| | Mean ± S.E. | |
|---|---|---|
| | Concentration (M) required to give half-maximum agonist response | Slope of log dose-response curve (mV/decade) |
| Dopamine (16) | $(3{\cdot}7 \pm 1{\cdot}1) \times 10^{-8}$ | $42{\cdot}7 \pm 3{\cdot}4$ |
| Noradrenaline (13) | $(1{\cdot}1 \pm 0{\cdot}3) \times 10^{-6}$* | $40{\cdot}2 \pm 3{\cdot}2$ |
| Adrenaline (10) | $(2{\cdot}4 \pm 0{\cdot}55) \times 10^{-6}$* | $69{\cdot}9 \pm 8{\cdot}3$* |
| 5-HT (11) | $(2{\cdot}7 \pm 0{\cdot}59) \times 10^{-7}$* | $30{\cdot}0 \pm 3{\cdot}3$* |

The number of experiments is given in parentheses after each agonist.

* Significantly different from dopamine value (group t test, $P < 0{\cdot}005$).

---

The average slope for adrenaline is steeper than for dopamine and noradrenaline, and this suggests that the receptor for adrenaline is different from the other catecholamine receptors.

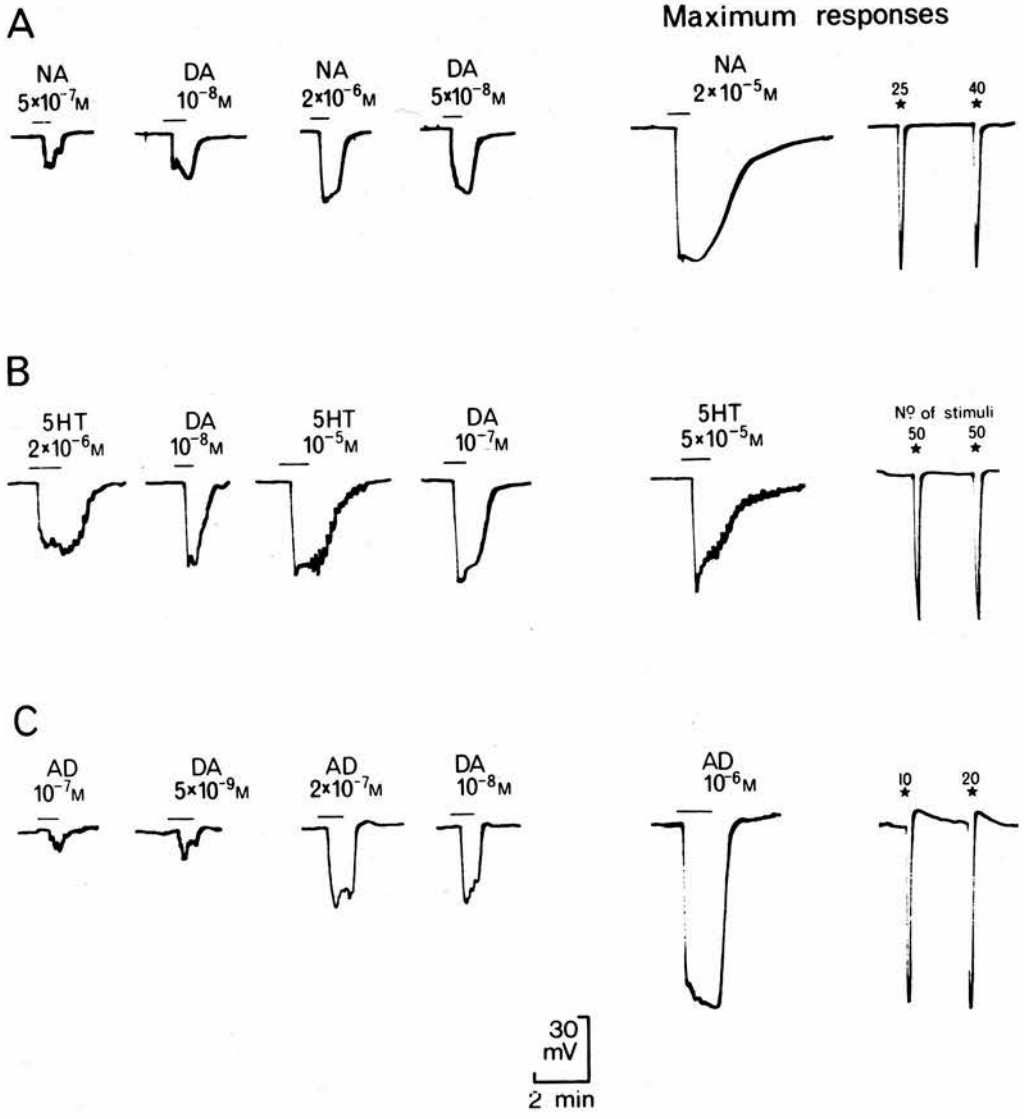
The conclusion that dopamine is more potent than

noradrenaline, adrenaline and 5-HT is supported by additional experiments where the effects of these agonists were compared in the same cell. For example, figure 23 shows recordings made in three different cells where equivalent responses to noradrenaline and dopamine were obtained in the first cell (23A) 5-HT and dopamine in the second (23B) and to adrenaline and dopamine in the third (23C). In 23A the dose ratios required for equal responses were about 50 NA: 1 DA, in 23C about 20 AD: 1 DA whereas in 23B they were about 150 5-HT: 1 DA. A further point of interest is that noradrenaline and adrenaline, like dopamine are able to elicit responses equal to the maximum neurally evoked responses whereas 5-HT cannot. In fact, the maximum 5-HT response was not maintained in this cell (fig 23B) although the duration of application was similar to that for the other agonists. The reason for this is not clear but it might be due to a greater desensitization to 5-HT than to the other agonists studied.

Another biogenic amine of possible significance is octopamine, which has been found in the nervous system of mammals (Molinoff & Axelrod, 1969), crustacea (Molinoff & Axelrod, 1972), molluscs (Walker, Ramage & Woodruff, 1972) and insects (Robertson & Steele, 1973). In contrast to the amines examined above, octopamine is a poor agonist, producing only a small hyperpolarization at a concentration of $10^{-5}$ M. Even at concentrations of $10^{-4}$ - $10^{-3}$ M it gave responses less than 25% of the

Figure 23

Comparison of responses to different biogenic amines recorded in the same cells. The duration of agonist application is indicated by the horizontal bars. (A) Responses to noradrenaline (NA) and dopamine (DA). Maximum responses to NA and nervous stimulation (number of stimuli above asterisks) are also shown. (B) Similar results for 5-HT and DA in a different cell. (C) Similar results for AD and DA also in a different cell. The neurally evoked maximal responses were obtained after the agonist responses and were equal to those (not shown) recorded at the beginning of the experiment. The resting potential of the cell in A was -30 mV, in B -34 mV and in C-35 mV.


A
Maximum responses
NA
5×10⁻⁷M
DA
10⁻⁸M
NA
2×10⁻⁶M
DA
5×10⁻⁸M
NA
2×10⁻⁵M
25
40
B
5HT
2×10⁻⁶M
DA
10⁻⁸M
5HT
10⁻⁵M
DA
10⁻⁷M
5HT
5×10⁻⁵M
Nº of stimuli
50
50
C
AD
10⁻⁷M
DA
5×10⁻⁹M
AD
2×10⁻⁷M
DA
10⁻⁸M
AD
10⁻⁶M
10
20
30
mV
2 min

neurally evoked maximum response (4 experiments). Applied at $10^{-7}$ M, octopamine increased the neurally evoked responses by about 30% and at $10^{-5}$ M the increase was followed by a pronounced decline during prolonged exposure (15 min). Octopamine's ability to alter the size of the neurally evoked response has not been investigated further.

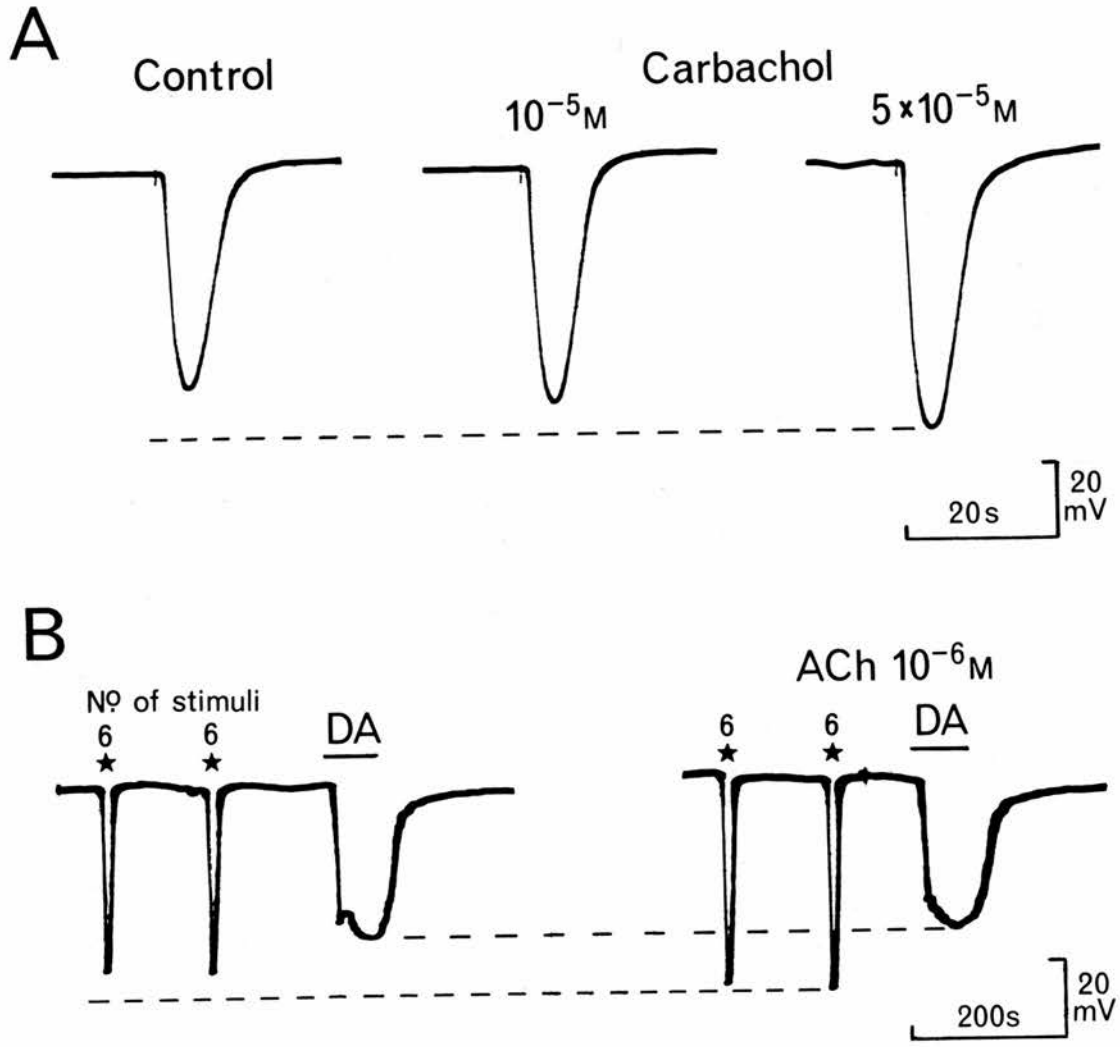
Actions of Acetylcholine and Carbachol.

In a previous study (Bowser-Riley, 1974) to test the actions of other known transmitter substances on the cockroach salivary gland it was found that only the synthetic cholinergic agonist carbachol was able to influence transmission at this neuroglandular junction. It was therefore thought worthwhile to extend these observations.

Neither ACh nor carbachol mimicked nerve stimulation even when applied at concentrations as high as $10^{-3}$ M. Carbachol, however, increased the neurally evoked response when its concentration was equal to or above $10^{-5}$ M (fig 24A). Above $5 \times 10^{-5}$ M it caused spontaneous hyperpolarizations with a similar time course to the hyperpolarizing potentials evoked by small numbers of stimuli. In the presence of the anticholinesterase, physostigmine, ACh also increased the neurally evoked response (fig 24B) and at higher concentrations it also elicited spontaneous potentials (fig 25 C,D) similar to those caused by carbachol. The recordings in figure 24B demonstrate that ACh can increase the neurally evoked response without changing the sensitivity of the cell to dopamine, since there was no increment in the submaximal dopamine responses. Thus it is plausible to suppose that ACh acts on the nerve terminals and enhances transmitter release. This proposal seems compatible with the occurrence of spontaneous potentials in the presence of ACh (fig 25 C,D) which are normally rarely observed in this gland (House, 1973).

Figure 24

Effects of carbachol and ACh on the neurally evoked responses of two cells. (A) Graded increase in the evoked responses (2 stimuli) in the presence of carbachol at two different concentrations. The resting potential was -40 mV. (B) Similar effect of ACh on the evoked response whereas the response to dopamine (DA) at $5 \times 10^{-8}$ M was unaffected. Physostigmine ($1{\cdot}3 \times 10^{-5}$ M) was present before and during ACh application. The resting potential of this cell was -28 mV.

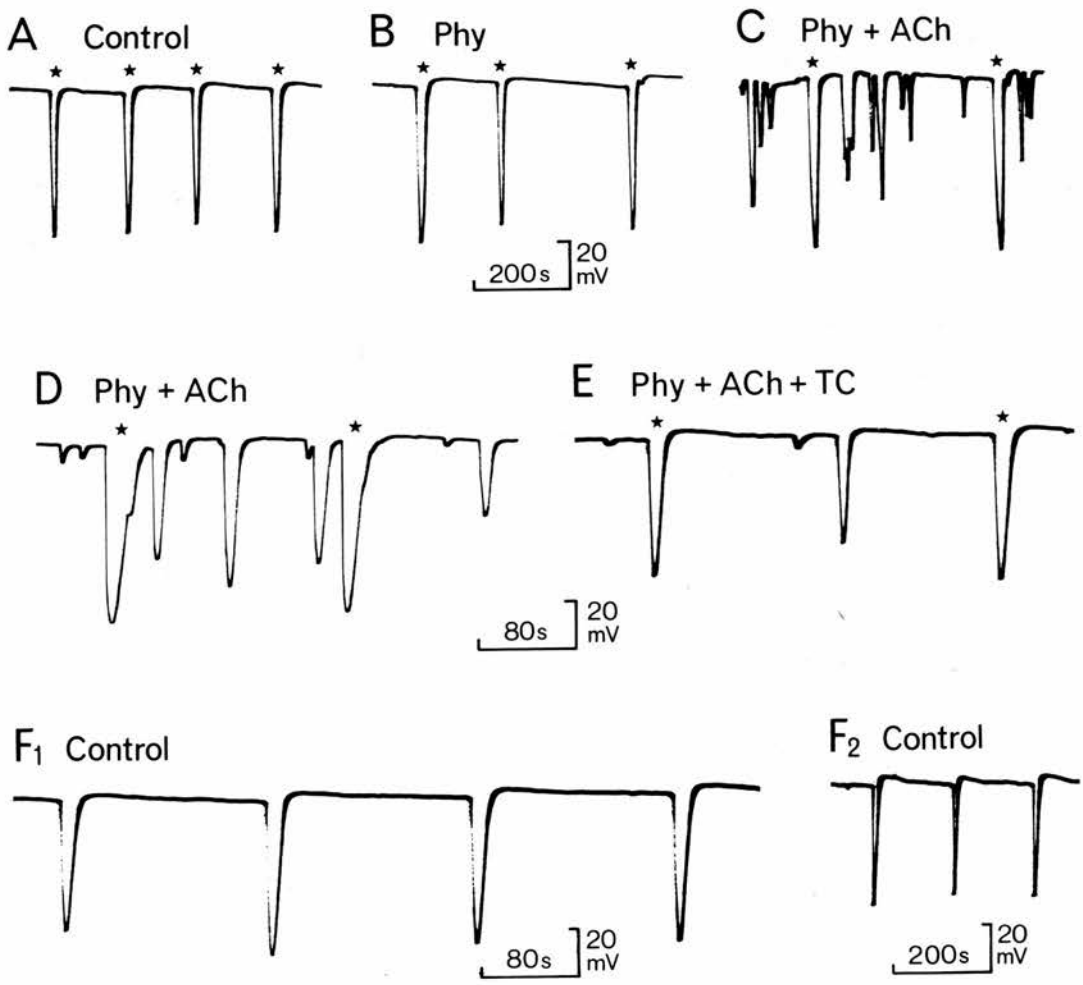

A
Control
Carbachol
$10^{-5}$M
$5 \times 10^{-5}$M
20s
20 mV
B
Nº of stimuli
6
6
DA
ACh $10^{-6}$M
6
6
DA
200s
20 mV

Nevertheless, other hypotheses are not excluded by these results, and the effects of ACh and carbachol require further study.

As ACh can influence transmission at this junction it was decided to investigate the effects of cholinergic blocking agents. Figure 25 displays the results of an experiment with (+)-tubocurarine. In order to obtain an effect from ACh the tissue was pretreated with physostigmine which itself produced a small increment in the amplitude of the neurally evoked responses (marked by asterisks) (cf 25 A,B). When ACh was applied at $10^{-4}$ M it caused a further increase in the neurally evoked response and also the emergence of spontaneous potentials (fig 25C,D). Prolonged exposure (20 - 30 min) to (+)- tubocurarine at $1{\cdot}3 \times 10^{-5}$ M abolished the increment in the neurally evoked response produced by ACh and also reduced the frequency and size of the spontaneous potentials (fig 25E). The neurally evoked responses recorded 5 - 15 min after washout of these drugs with physiological saline (fig $25F_1$) were similar to those observed initially (25A); moreover, the preparation was not affected adversely by these substances as judged by the responses recorded 30 - 35 min after washout ($25F_2$). In the absence of physostigmine, ACh failed to generate the effects shown in figs 24 and 25 even at concentrations as high as $10^{-3}$ M. These experiments suggest that there are cholinergic receptors, possibly nicotinic, in the salivary nerve terminals. The release of transmitter,

Figure 25

Actions of ACh and (+)-tubocurarine (TC) in the presence of physostigmine (Phy) on the neurally evoked responses of an acinar cell. The responses evoked by single stimuli are marked by asterisks. Unmarked changes in potential are spontaneous. (A - F) Extracts from a continuous record. B shows that physostigmine (Phy) at $1{\cdot}3 \times 10^{-5}$ M, applied 12 min previously at the end of A, produced a small increase in the evoked response, C and D illustrate that ACh ($10^{-4}$ M) produced a further increase in the evoked response accompanied by spontaneous potentials, these traces being obtained 8 and 33 min after the end of B. E shows that the addition of TC at $1{\cdot}3 \times 10^{-5}$ M markedly reduced the effects produced by ACh + Phy, this trace being obtained 50 min after the end of D. F Evoked potentials recorded 5 - 15 min ($F_1$) and 30 - 35 min ($F_2$) after washout with physiological saline.

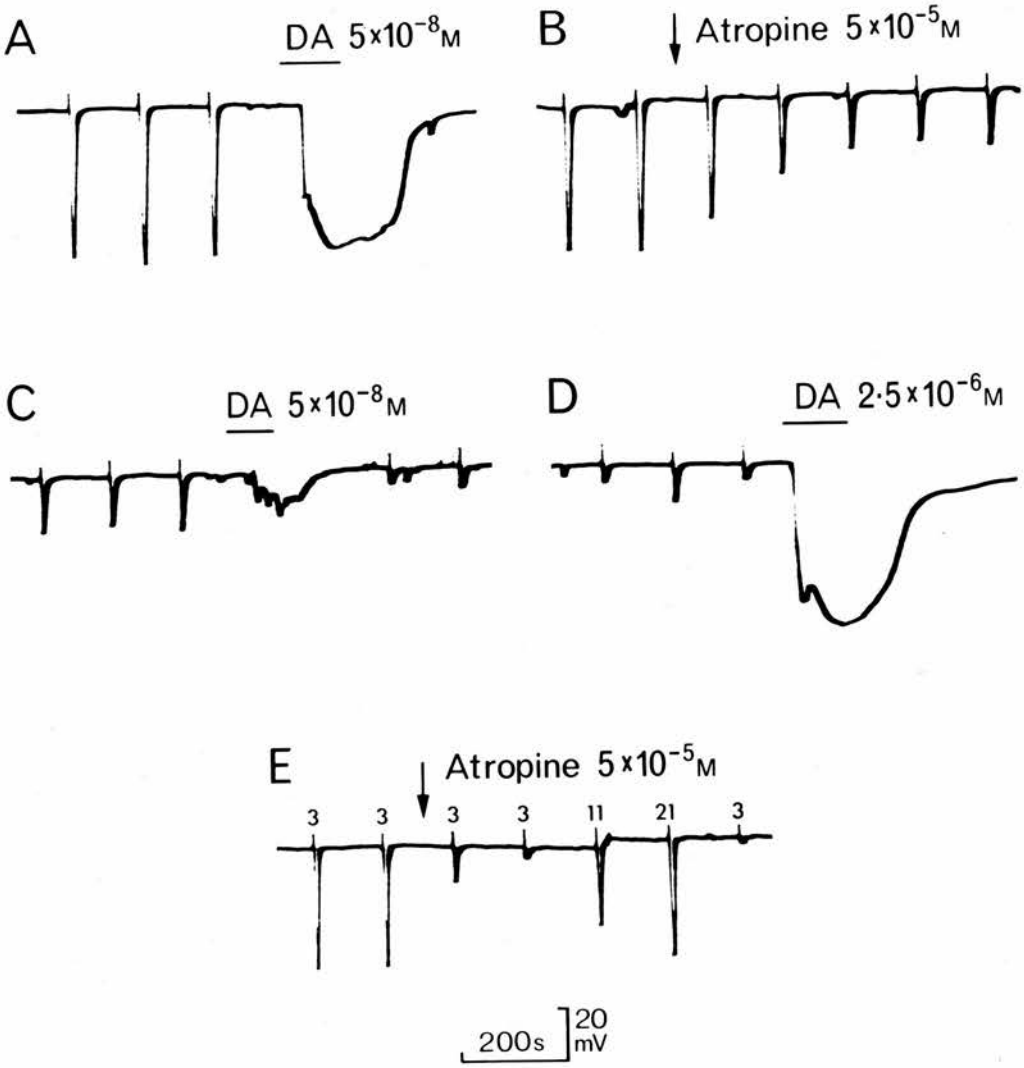

A Control
B Phy
C Phy + ACh
200s
20 mV
D Phy + ACh
E Phy + ACh + TC
80s
20 mV
F1 Control
F2 Control
80s
20 mV
200s
20 mV

however is not obligatorily dependent on the activation of these receptors since two further experiments also demonstrated that (+)-tubocurarine did not abolish the evoked response. It was therefore of interest to examine the effects of a muscarinic blocker. Atropine was found to reduce the size of the neurally evoked response (fig 26A,B). However, the response to dopamine was also diminished (24A,C), but could be restored to its original magnitude by a 50-fold increase in the dopamine concentration. This suggests that atropine acts postjunctionally in this preparation probably in a competitive manner, its affinity constant for the dopamine receptors being about $10^{-6}$M. This is roughly, 100-1000 times less than values for muscarinic receptors in mammalian preparations (see Barlow, 1964). Additional evidence suggesting that the action of atropine on the neurally evoked response is also postjunctional was obtained in three experiments where it was found that atropine block could be surmounted by increasing the number of nerve stimuli (fig 26E). The possibility that atropine acts as a local anaesthetic at the concentrations (ca. $5 \times 10^{-5}$) used in these experiments has not been excluded, although it seems unlikely in view of its failure at $10^{-4}$ M to reduce the size of the action potential of mammalian C fibres (Armett & Ritchie, 1961).

Thus atropine can substantially reduce the neurally evoked response probably by interacting with the gland cell receptors for the transmitter. In contrast, it is likely that tubocurarine blocks cholinergic receptors

Figure 26

The blocking action of atropine on the dopamine and neurally evoked responses of two acinar cells. The records A to D are consecutive, the application of dopamine (DA) is indicated by the bars and atropine is present from the arrow in trace B, through C and D; the neurally evoked responses were elicited by three stimuli throughout. E shows that the atropine blockade of the neurally evoked response can be surmounted by increasing the number of nerve stimuli in the volley; the number of stimuli is indicated above each response. The resting potential in each case was -24 mV.


A
DA 5×10⁻⁸M
B
Atropine 5×10⁻⁵M
C
DA 5×10⁻⁸M
D
DA 2·5×10⁻⁶M
E
Atropine 5×10⁻⁵M
3
3
3
3
11
21
3
200s
20
mV

on salivary nerve terminals.

Actions of Phentolamine.

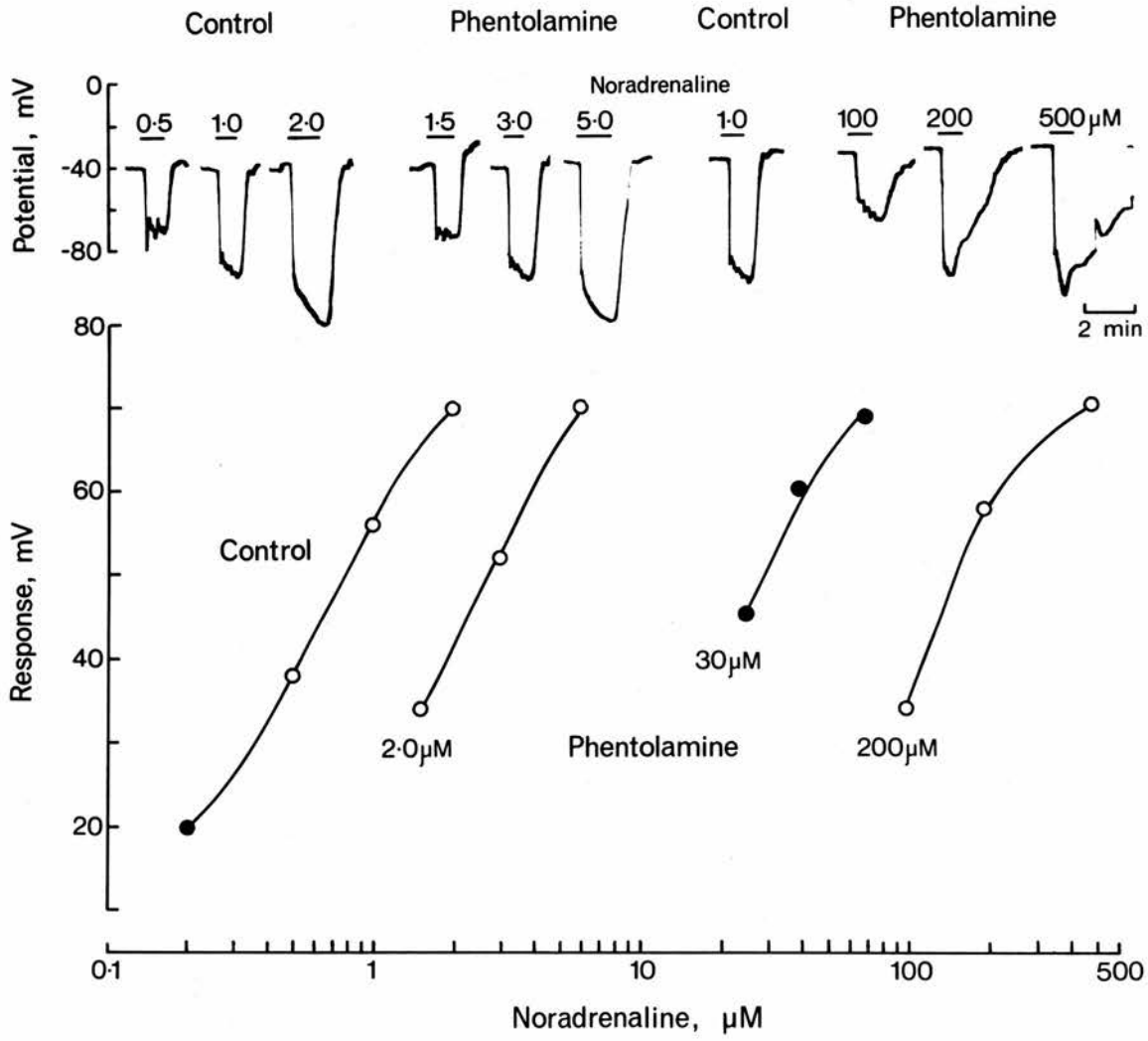
The results of this section concern the blocking action of phentolamine on the dose-dependent hyperpolarization induced by bath application of the biogenic amines and nervous stimulation.

If the antagonism exerted by phentolamine is competitive, as suggested by the results of Ginsborg et al. (1976), the dose response curves for the biogenic amines should, according to equation (1), be shifted to the right without change in their amplitude or slope. The magnitude of this shift (i.e. the dose ratio, x) should be linearly related to the antagonist concentration, I, where K, the affinity constant is independent of antagonist concentration (Furchgott, 1972).

The action of phentolamine was studied by monitoring its effects on the dose-response relationships for each agonist in the presence of various concentrations of this inhibitor. An example of the general experimental format is shown in figure 27. The responses evoked by the bath applied agonist, which in this case was noradrenaline, are illustrated in the upper part and the corresponding log dose-response curves in the lower part of the figure. Clearly phentolamine caused a parallel shift of the dose-response curve for this agonist. Similarly it was found that the dose-response curves for adrenaline (fig 28), dopamine (fig 29), and 5-HT (fig 30) were also displaced in a parallel fashion in the presence of this agonist. Other experiments indicated that the parallel displacement

Figure 27

Antagonism by phentolamine of the electrical response of an acinar cell to noradrenaline. Upper part shows some intracellular recordings of the hyperpolarizing responses to noradrenaline before and during the successive application of 2·0 and 200 $\mu$M phentolamine, the larger concentration requiring the higher equipotent dose ratio of the agonist. The experimental results are plotted as log dose-response curves in the lower part of the figure with the corresponding phentolamine concentration below each curve. The open circles denote the values of the hyperpolarizing response shown above. The control response to 1·0 $\mu$M noradrenaline represents the recovery after washoff of 2·0 $\mu$M phentolamine; a similar value was obtained following the washoff of 30 $\mu$M phentolamine. No recovery was observed from the 200 $\mu$M phentolamine since the electrode was lost from the cell during the application of 500 $\mu$M noradrenaline.


Control
Phentolamine
Control
Phentolamine
Noradrenaline
Potential, mV
0
-40
-80
0·5
1·0
2·0
1·5
3·0
5·0
1·0
100
200
500 μM
2 min
Response, mV
80
60
40
20
Control
2·0μM
30μM
Phentolamine
200μM
0·1
1
10
100
500
Noradrenaline, μM

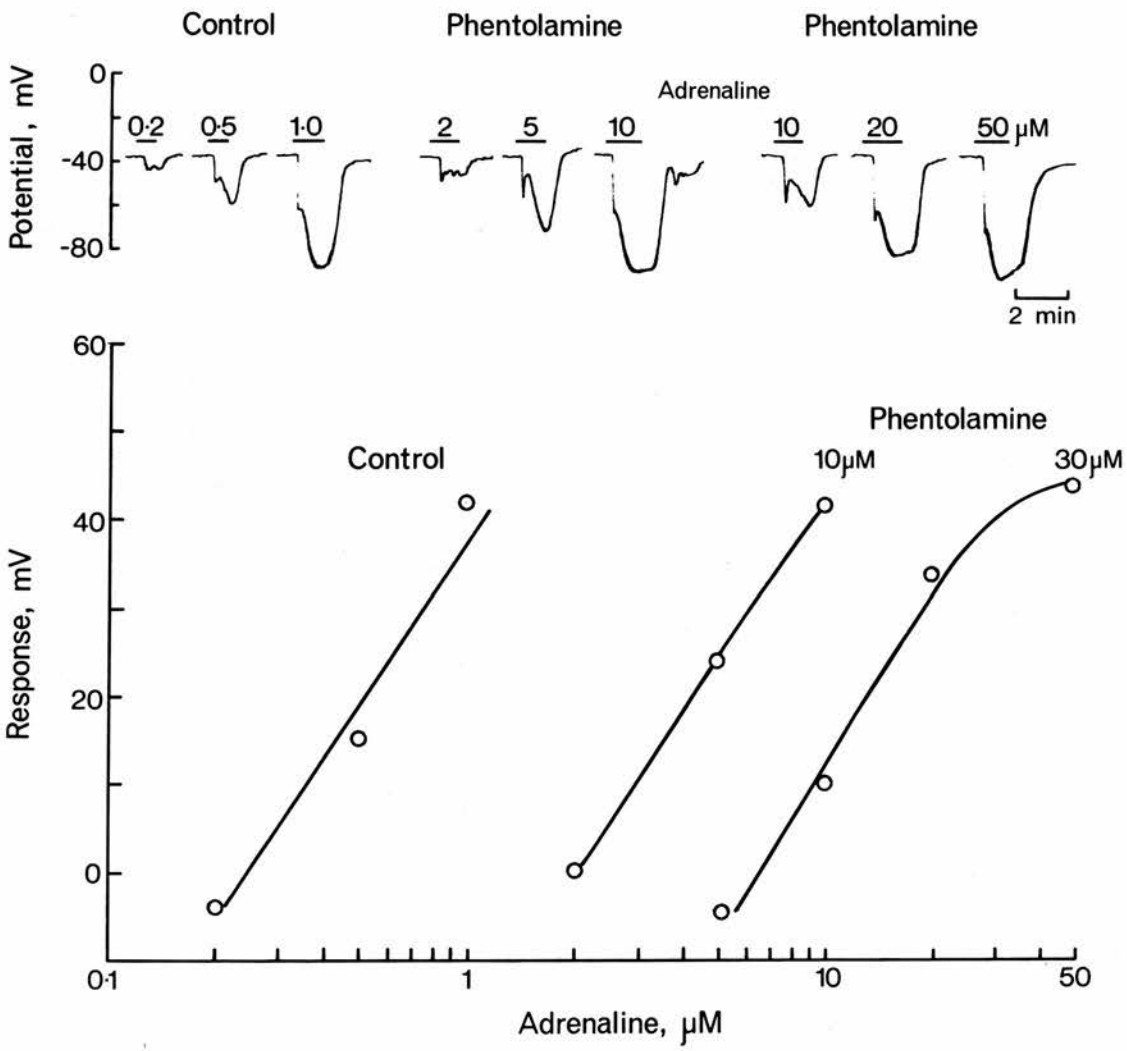
was proportional to the phentolamine concentration. Attempts to match maximal responses were successful for all agonists except 5-HT (see later).

These results suggest that there is a competitive antagonism between phentolamine and the catecholamines. Thus from equation (1) it should be possible to estimate the affinity constant, K, of this inhibitor for the receptors involved by obtaining matching agonist responses before and during its application. The method of equal responses, i.e. a null experiment, avoids any assumption about the relation between the amplitude of the response and the fraction of receptors occupied and thus allows the validity of equation (1) to be examined. Null experiments however, generally yield results which are also virtually identical to the predictions of various co-operative models (see Colquhoun, 1975). Nevertheless, it seems more appropriate to this study to adopt the simpler classical view of antagonism.

The results illustrated in figure 27 permit one to calculate the affinity constant for phentolamine's antagonism of the noradrenaline-evoked response. In this experiment it was possible to obtain three successive dose-response curves for noradrenaline in the presence of three different concentrations of phentolamine, 2, 30 and 200 $\mu$M. The dose-response curves were parallel and the equipotent dose ratios were 3·3, 25 and 260 giving affinity constants of 1·2, 0·8 and 1·3 $(\mu M)^{-1}$ respectively. In another experiment where successive

Figure 28

Antagonism by phentolamine of the electrical response of an acinar cell to adrenaline. Upper part shows some intracellular recordings of the hyperpolarizing responses to adrenaline before and during the successive application of 10 and 30 $\mu$M phentolamine. The experimental results are plotted as log dose-response curves in the lower part of the figure with the corresponding phentolamine concentration above each curve.


Control
Phentolamine
Phentolamine
Adrenaline
Potential, mV
0
-40
-80
0·2
0·5
1·0
2
5
10
10
20
50 μM
2 min
60
40
20
0
Response, mV
Control
Phentolamine
10μM
30μM
0·1
1
10
50
Adrenaline, μM

dose-response curves for adrenaline (fig 28) were obtained in the presence of 10, 30 and 100 $\mu$M phentolamine giving equipotent dose ratios of 9·5, 28 and 120 the calculated affinity constants were 0·85, 0·9 and 1·2 $(\mu M)^{-1}$ respectively. Similar experiments were made for the other agonists, dopamine (fig 29) and 5-HT (fig 30) however in these and other experiments with all the agonists only single values for the affinity constant for phentolamine at a given concentration were obtained since the period of stable recording was invariably too short. The value of the affinity constant for the experiment shown in figure 29 of phentolamine's inhibition of the dopamine response was 0·35 $(\mu M)^{-1}$ where the antagonist concentration was 20 $\mu$M and the equipotent dose ratio was 8. That calculated for the antagonism of 5-HT (fig 30) was 0·01 $(\mu M)^{-1}$.

The estimates of K obtained over a 100 fold change in phentolamine concentration for the inhibition of the agonist response is shown in table 3. There was no significant difference between the affinity constants obtained in the experiments with dopamine, noradrenaline and adrenaline. It is clear that the estimates of K for the catecholamine receptors are independent of phentolamine concentration over a wide range (2 - 500 $\mu$M). However the affinity constant of phentolamine acting on the 5-HT receptors was significantly different being about 100-times smaller than the other estimates. High concentrations of phentolamine were required for

Figure 29

Antagonism by phentolamine of the electrical response of an acinar cell to dopamine. Upper part shows some intracellular recordings of hyperpolarizing responses to dopamine before and during the application of 20 $\mu$M phentolamine. The experimental results are plotted as log dose-response curves in the lower part of the figure.

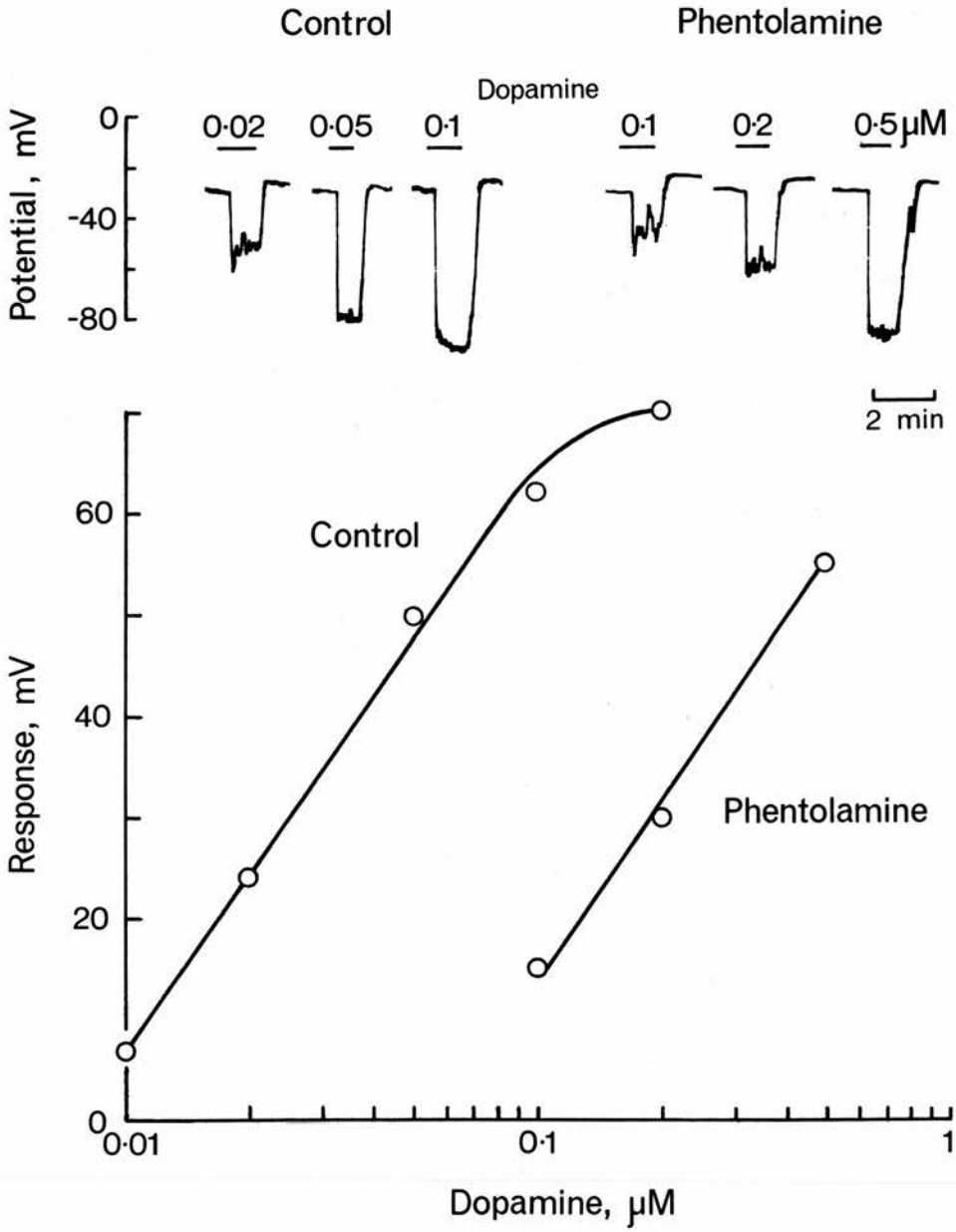

Control
Phentolamine
Dopamine
0·02
0·05
0·1
0·1
0·2
0·5μM
Potential, mV
0
-40
-80
2 min
Control
Phentolamine
Response, mV
60
40
20
0
0·01
0·1
1
Dopamine, μM

Figure 30

Antagonism by 20 $\mu$M phentolamine of the electrical response of an acinar cell to 5-HT. The experimental results have been plotted as log dose-response curves and extracts of the hyperpolarizing response before and during the application of phentolamine are shown in the following figure (fig 31A).

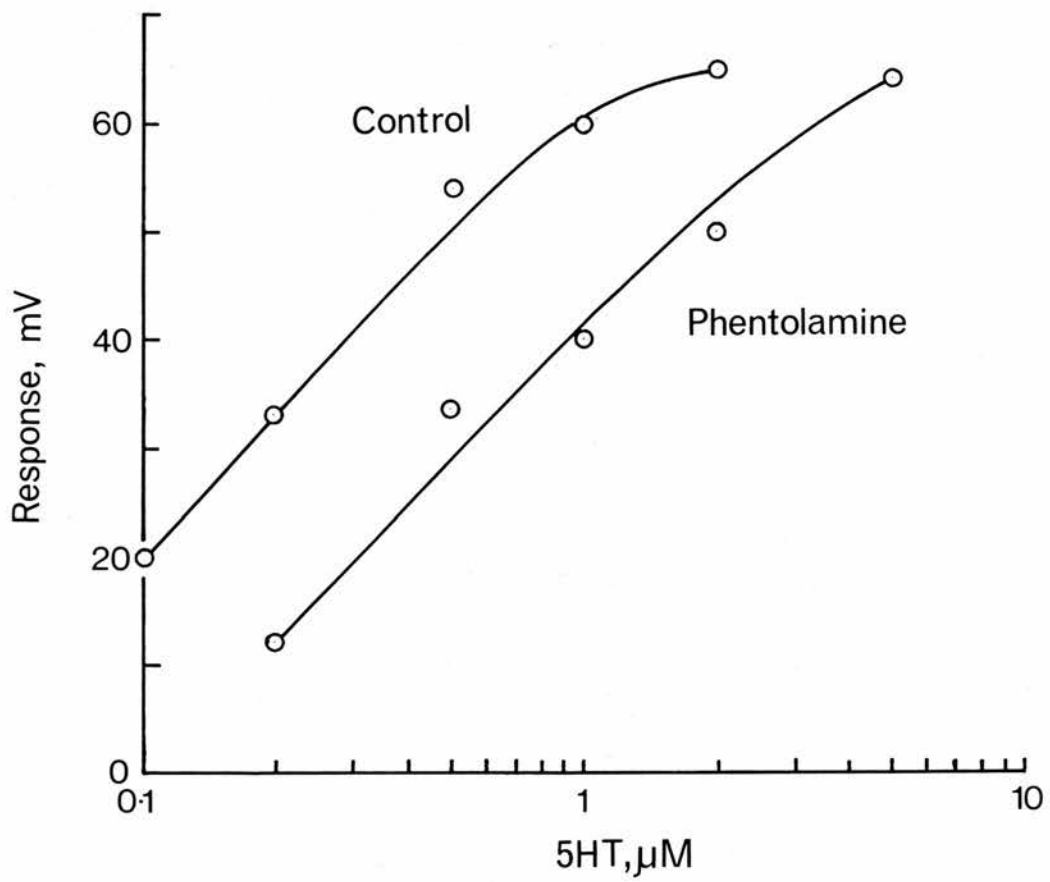

Control
Phentolamine
Response, mV
60
40
20
0
0·1
1
10
5HT, μM

TABLE 3

Estimates of affinity constants from experiments on phentolamine's inhibition of electrical responses.

| Phentolamine Conc. ($\mu$M) | Equipotent Dose-ratio | Affinity constant ($\mu$M)$^{-1}$ |
|---|---|---|
| | **DOPAMINE** | |
| 2 | 3·0 | 1·0 |
| 5 | 6·0 | 1·0 |
| 10 | 10 | 0·90 |
| 20 | 8·0 | 0·35 |
| 30 | 130 | 4·3 |
| 30 | 100 | 3·3 |
| 60 | 64 | 1·1 |
| 100 | 70 | 0.69 |
| 500 | 500 | 1·0 |
| | mean $\pm$ S.E. | 1·5 $\pm$ 0·4 |
| | **NORADRENALINE** | |
| 2 | 3·3 | 1·2* |
| 5 | 8·3 | 1·5 |
| 10 | 7·4 | 0·64 |
| 10 | 10 | 0·90 |
| 30 | 25 | 0·80* |
| 50 | 150 | 3·0 |
| 100 | 300 | 3·0 |
| 200 | 260 | 1·3* |
| | mean $\pm$ S.E. | 1·5 $\pm$ 0·3 |
| | **ADRENALINE** | |
| 3 | 3·5 | 0·83 |
| 10 | 9·5 | 0·85* |
| 30 | 28 | 0·90* |
| 100 | 120 | 1·2* |
| 100 | 90 | 0·89 |
| | mean $\pm$ S.E. | 0·93 $\pm$ 0·07 |

* Obtained in the same cell for each agonist.

cont. over

TABLE 3 (cont.)

Estimates of affinity constants from experiments on phentolamine's inhibition of electrical responses.

| Phentolamine Conc. ($\mu$M) | Equipotent Dose-ratio | Affinity constant ($\mu$M)$^{-1}$ |
|---|---|---|
| | NERVE | |
| 1 | 1·7 | 0·70 |
| 1 | 1·7 | 0·70 |
| 1 | 1·6 | 0·60 |
| 2 | 4·3 | 1·7 |
| 3 | 7·0 | 2·0 |
| 3 | 2·1 | 0·37 |
| 5 | 2·5 | 0·30 |
| 5 | 4·3 | 0·66 |
| 5 | 6·7 | 1·1 |
| 5 | 4·5 | 0·70 |
| 7 | 4·5 | 0·50 |
| 10 | 5·5 | 0·45 |
| 10 | 15 | 1·4 |
| 30 | 50 | 1·6 |
| | mean ± S.E. | 0·91 ± 0·14 |
| | 5-HYDROXYTRYPTAMINE | |
| 50 | 2·0 | 0·020 |
| 100 | 2·9 | 0·019 |
| 200 | 3·0 | 0·010 |
| 500 | 5·2 | 0·008 |
| 500 | 10 | 0·018 |
| 600 | 12 | 0·018 |
| 600 | 8·2 | 0·012 |
| | mean ± S.E. | 0·015 ± 0·002 ∓ |

∓ Significantly different from other means (group t test, $P \leq 0{\cdot}01$).

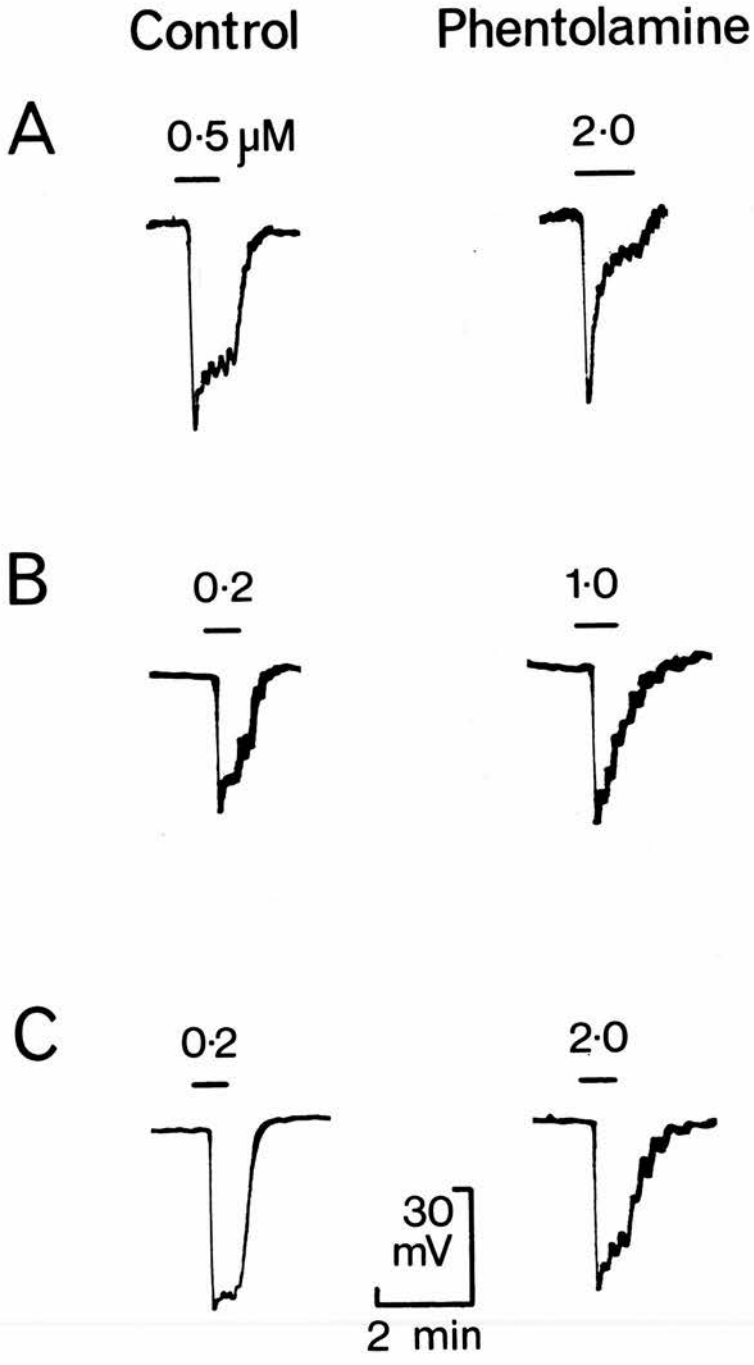
suppression of the 5-HT response and yet K was apparently independent of concentration over a tenfold range. Above 600 $\mu$M, however, estimates of K exceeded the values in table 3 and increased proportionally with inhibitor concentration. It was also observed that the responses to 5-HT fell more rapidly in the presence of high concentrations of phentolamine (fig 31). A similar but slower fall in the response in the absence of this inhibitor observed previously (cf fig 23) was suggested to be due to desensitization of the 5-HT receptors and in this case may arise from an acceleration of this process.

It was of interest to ascertain whether the antagonism of the neurally evoked response could be investigated quantitatively, since if estimates of the affinity constant for a given antagonist acting on the same receptor are identical this would indicate that the two responses are mediated by the same receptor (Schild, 1947).

This required a method of obtaining equipotent dose ratios in conformity with the approach based on equation (1). The previous evidence that the amplitude of the hyperpolarization is graded with the number of stimuli in a dose dependent fashion suggested that a null experiment might be possible. Figure 32 shows a plot of the peak values of the hyperpolarizing response against the logarithm of the number of stimuli, where the slope is similar to that of the noradrenaline dose response curve obtained in the same cell. Accurate superimposition was also possible for nerve and dopamine

Figure 31

Action of phentolamine on the time course of the electrical responses of acinar cells to 5-HT. Three representative intracellular recordings from different preparations are shown in A, B and C where the resting potentials were -40, -34 and -36 respectively. The 5-HT concentrations applied are indicated above the horizontal (duration) bars. The phentolamine concentrations were 200, 500 and 600 $\mu$M in A, B and C respectively and in each case caused a more rapid fall in the response than that observed in the corresponding controls.


Control
Phentolamine
A
0·5 μM
2·0
B
0·2
1·0
C
0·2
2·0
30
mV
2 min

stimulation (fig 33) where electrical responses were produced in the same cell. In this case an additional dose response curve to 5-HT was also obtained whilst recording from the same cell. Clearly it is not possible to superimpose the values for nerve on the 5-HT curve since the response to this agonist is always less than the maximum to nerve stimulation and the slope of its dose response curve was too shallow.

The fact that the dose-response curves for the nerve response and dopamine or noradrenaline can be superimposed may be entirely fortuitous. It is possible, however, that the similarity indicates that the local concentration of the transmitter at the neuroglandular junction is proportional to the number of stimuli over a modest range. Although there is no direct evidence to support this it seems worthwhile to take this as a working hypothesis to explore the consequences concerning the action of phentolamine at this neuroglandular junction.

Consequently attempts were made to measure the affinity constant along the previous lines with the modification that the equipotent dose ratio was estimated as the ratio of number of stimuli required for matching responses. An important precondition was that a single stimulus should produce a large response i.e. $> 20$ mV. There were two reasons for this condition. First, it avoided non-linearities in the stimulus-response curve for small numbers (cf fig 19). Secondly it meant that the required matching response in the presence of

Figure 32

Stimulus-response relationships of an acinar cell for the hyperpolarizing responses to nerve stimulation and noradrenaline application. Intracellular recordings of some of these responses are shown above the corresponding log dose-response (o) and log stimulus number-response (•) curves. These curves have been superimposed by appropriate positioning of the abscissae. The number of nerve stimuli and noradrenaline concentrations are given above the responses which have been extracted from a continuous record of membrane potential.

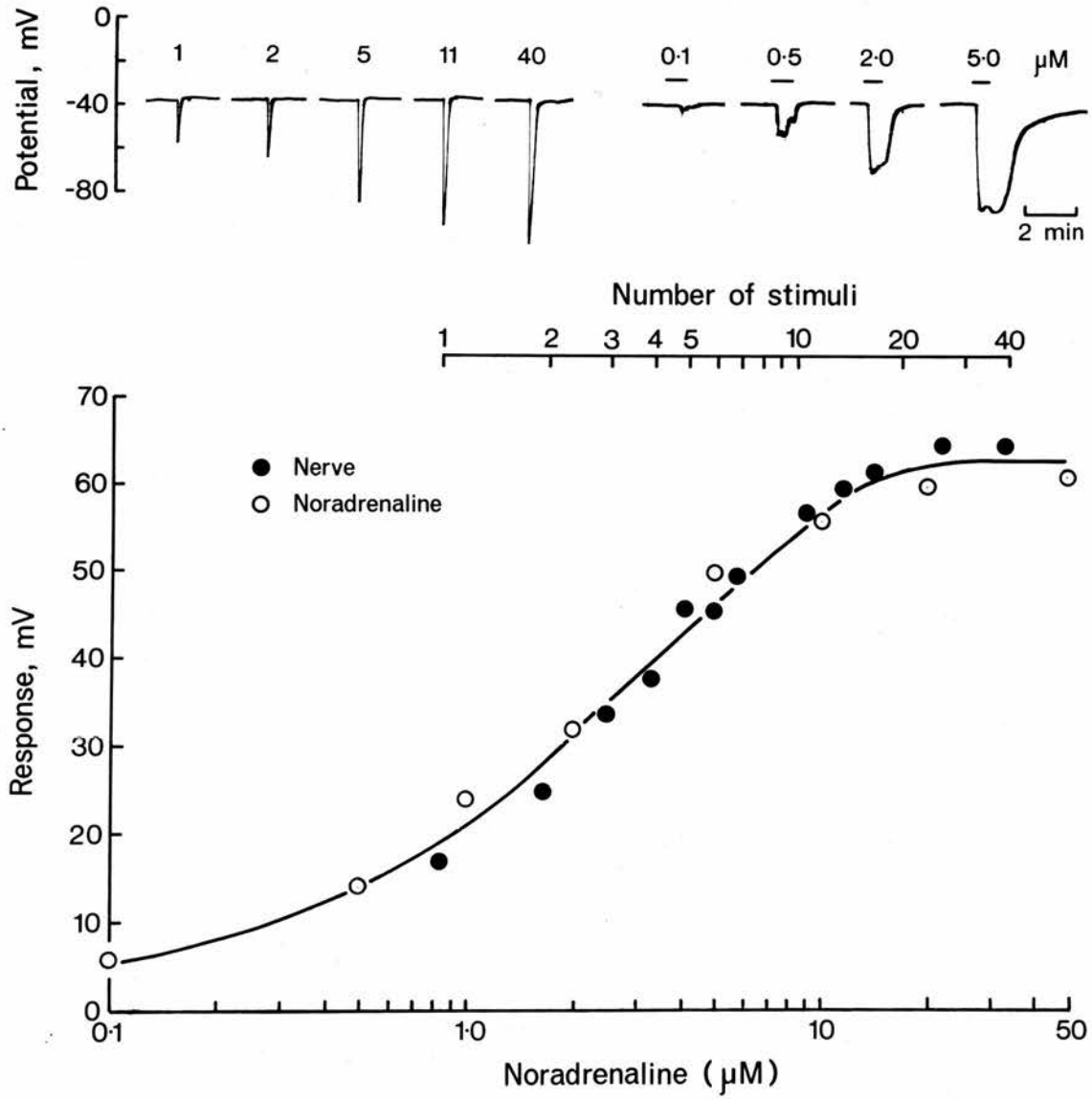
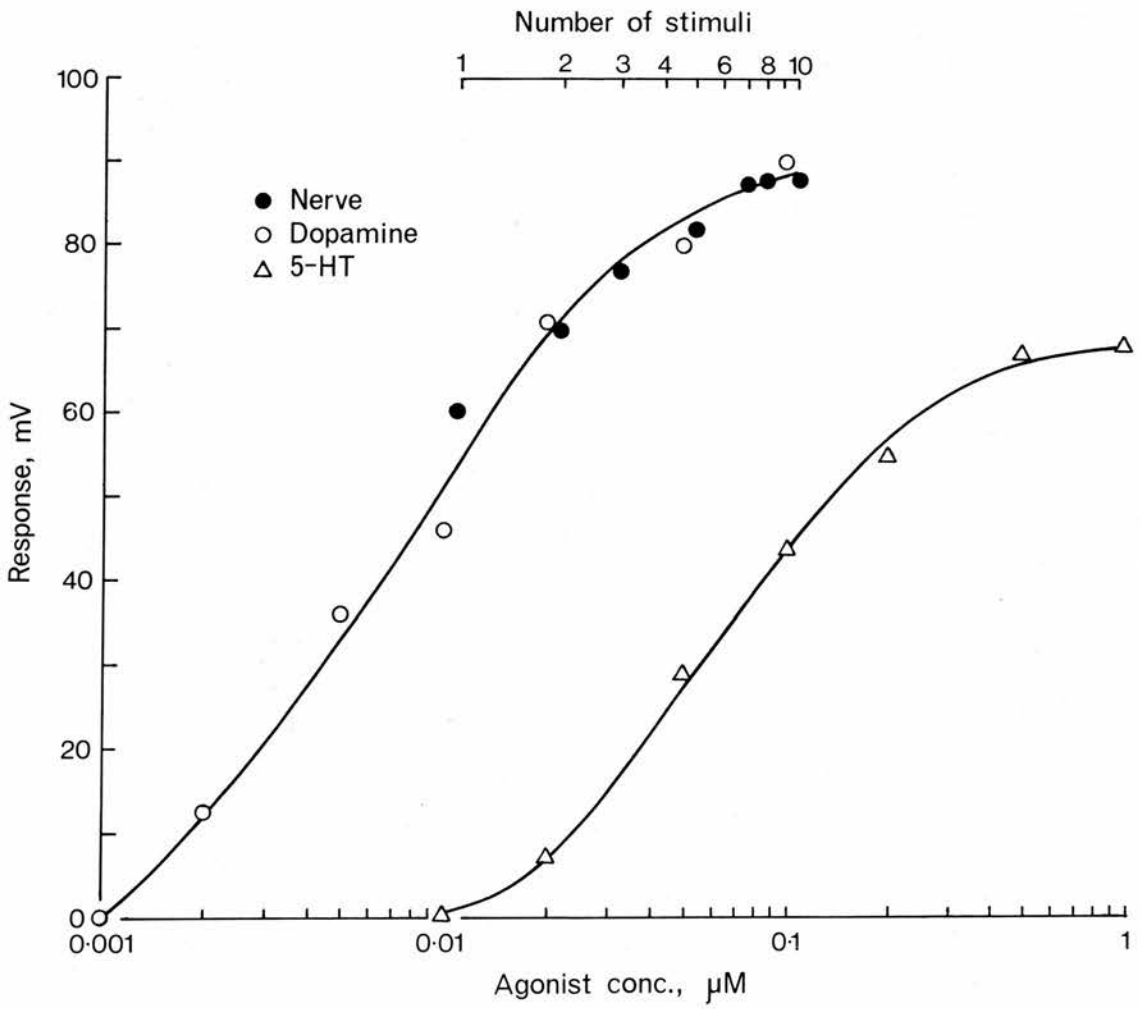

Potential, mV
0
-40
-80
1
2
5
11
40
0·1
0·5
2·0
5·0
μM
2 min
Number of stimuli
1
2
3
4
5
10
20
40
Response, mV
70
60
50
40
30
20
10
0
Nerve
Noradrenaline
0·1
1·0
10
50
Noradrenaline (μM)

Figure 33

Stimulus-response curves for the hyperpolarizing responses induced by nerve stimulation (●) and dopamine (o) and 5-HT (△) application, obtained whilst recording from the same acinar cell. The curve for the neural response has been superimposed on the dopamine curve by appropriate positioning of the abscissae. Clearly the neural response curve cannot be superimposed on the 5-HT since the maximal response obtained by the former exceeds that of the latter by about 20 mV. The resting potential of this cell was -34 mV.

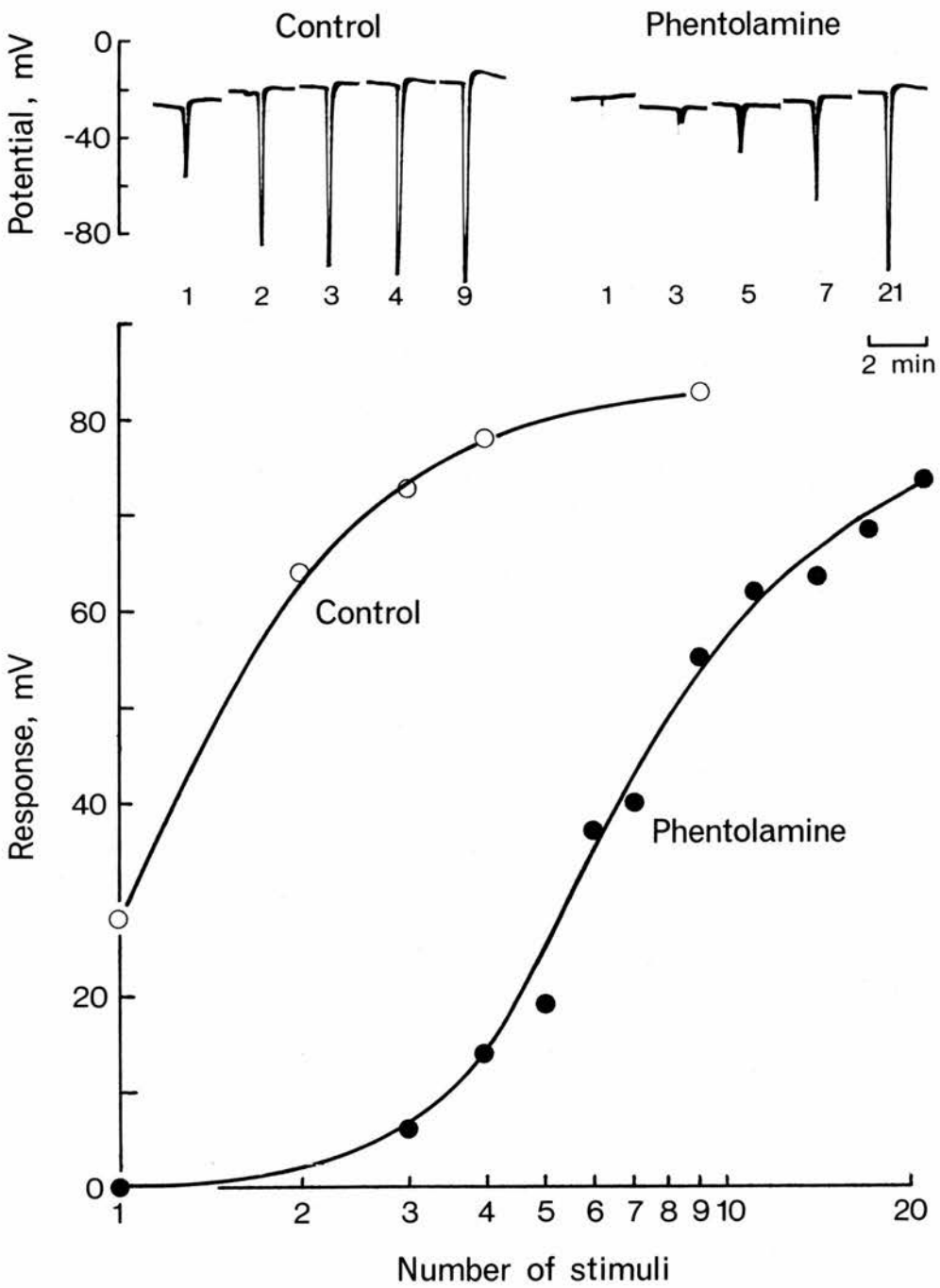

Number of stimuli
1
2
3
4
6
8
10
100
80
60
40
20
0
Response, mV
Nerve
Dopamine
5-HT
0·001
0·01
0·1
1
Agonist conc., μM

the antagonist could be evoked without having to deliver large numbers of stimuli, i.e. >200. Provided that this precondition was satisfied it was possible to achieve matching responses and a typical experiment is shown in figure 34; intracellular recordings are shown above the stimulus-response curve. Evidently phentolamine caused a parallel shift of the curve, the equipotent number ratio being about 5, since the concentration of phentolamine was 10 $\mu$M the affinity constant was calculated as 0·4 $(\mu M)^{-1}$. Thirteen similar experiments were performed at different inhibitor concentrations (1 - 30 $\mu$M). These gave values of K in the range 0·3 - 2·0 $(\mu M)^{-1}$ (shown in table 3). It was not possible to do satisfactory experiments at concentrations above 30 $\mu$M since the maximum response could not be matched even with large numbers of stimuli (>500). It was considered, however, that transmitter output per stimulus would decline towards the end of such trains and hence invalidate the main assumption of the working hypothesis. Indeed there is evidence for a large fall in noradrenaline release from the sympathetic fibres as the number of stimuli approaches 1000 (Stjarne, Hedquist & Bygdeman, 1969). In view of that limitation an essential condition for successful experiments was that single stimuli should evoke a large response.

For an antagonist which acts competitively it is expected, according to equation (1), that there should be a linear relation with unit slope between log (x-1)

Figure 34

Antagonism by phentolamine of the hyperpolarizing response of an acinar cell to nerve stimulation. Intracellular recordings of some of the responses are illustrated in the upper part, the number of stimuli being given below each response. The corresponding log stimulus-response curves before and during the application of 10 $\mu$M phentolamine are shown below.

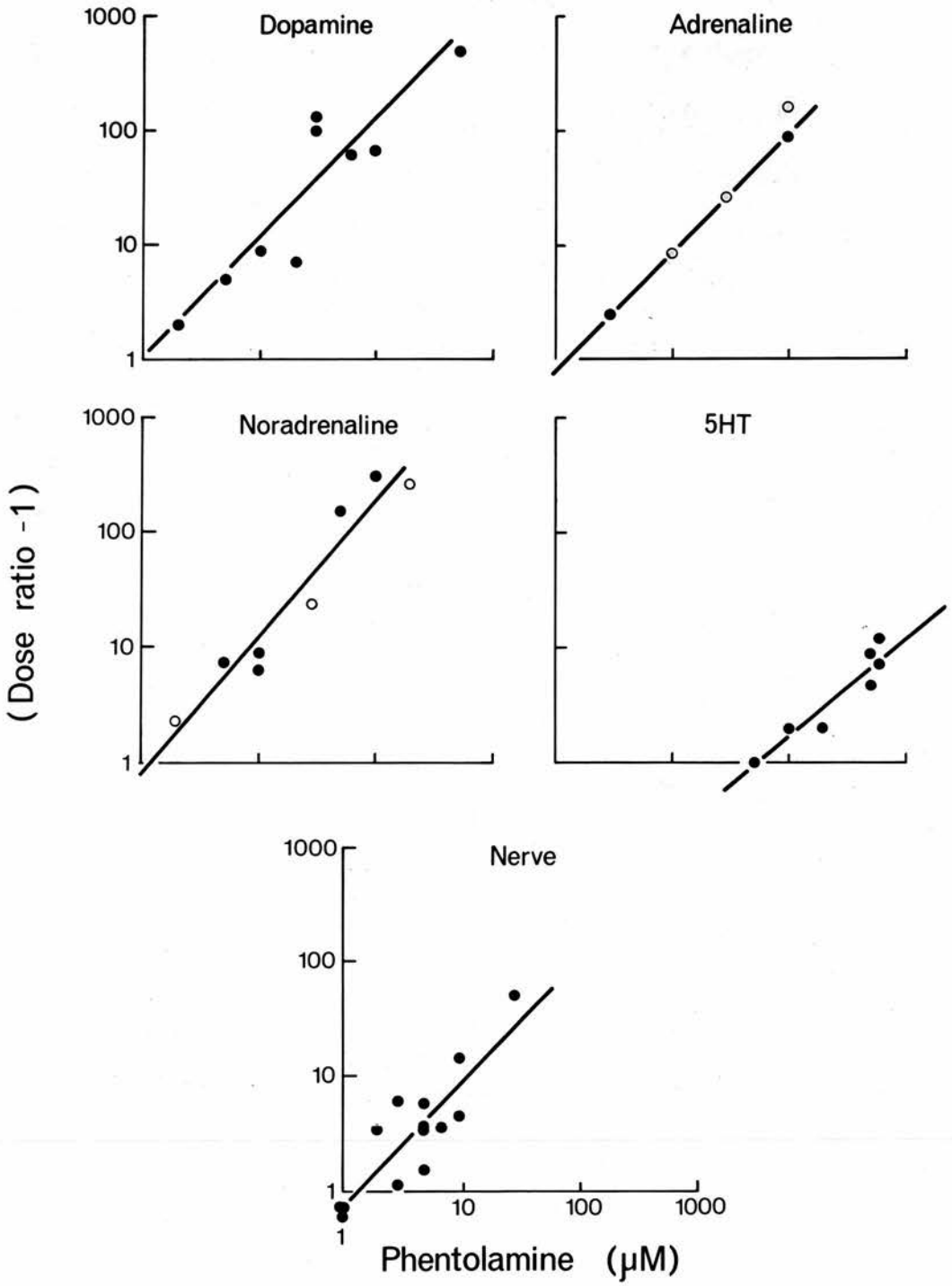

Control
Phentolamine
Potential, mV
0
-40
-80
1 2 3 4 9
1 3 5 7 21
2 min
Response, mV
80
60
40
20
0
Control
Phentolamine
1 2 3 4 5 6 7 8 9 10 20
Number of stimuli

and the log of the antagonist concentration (I) and when log (x-1) is zero (where dose ratio = 2) (I) is equal to $1/K_I$ (see Furchgott, 1972). Generally it was not feasible to test this relation in individual preparations because of the difficulties of maintaining stable recording conditions for the long experimental period required. In two experiments, however, the effects of different concentrations of phentolamine were studied in the same preparation. The affinity constants (marked by asterisks in table 3) were independent of phentolamine concentration thus upholding the validity of applying equation (1) to these results. Furthermore, the general features of the affinity constant measurements obtained in the other experiments are at least compatible with equation (1) as figure 35 shows. In this figure the values of log (x-1) have been plotted against the corresponding log value of the phentolamine concentration from the data given in table 3.

A least squares regression analysis (see Colquhoun, 1971) of the results for the catecholamines and the neurotransmitter confirmed that the slopes were close to unity (fig 35) and the values of K, ($(\mu M)^{-1} \pm$ S.E.*) computed from the intercept of the regression line with the x-axis (when log (x-1) = 0) were 1·09 ± 0·19, 0·81 ± 0·1, 0·77 ± 0·12 and 0·71 ± 0·17 for phentolamine's inhibition of the dopamine, noradrenaline, adrenaline and neurally evoked responses respectively. There was no significant difference (Student's t test) between

Figure 35

**Analysis of phentolamine's inhibition of the neurally evoked and agonist evoked responses. Each graph shows the combined results from the experiments on the hyperpolarizing responses to nerve stimulation and the agonists indicated. Each symbol denotes the results of a single experiment except for o which indicates measurements obtained on the same cell. The plots have been made to test the validity of equation (1), namely x-1 = K(I), where x = equipotent dose ratio and (I) = phentolamine concentration. The lines are computed regressions from the data and the slope of each is close to unity as predicted by equation (1). The computed slopes ($\pm$ S.E.) for the responses to dopamine, noradrenaline, adrenaline, 5-HT and nerve stimulation are 1·01 $\pm$ 0·18, 1·14 $\pm$ 0·14, 1·06 $\pm$ 0·04, 0·85 $\pm$ 0·14 and 1·09 $\pm$ 0·17 respectively. The computed values for K ($(\mu M)^{-1}$ $\pm$ S.E.) from the intercept of the regression line with the x axis are 1·09 $\pm$ 0·19, 0·81 $\pm$ 0·1, 0·77 $\pm$ 0·12, 0·018 $\pm$ 0·007 and 0·71 $\pm$ 0·17 for phentolamine's antagonism of the dopamine, noradrenaline, adrenaline, 5-HT and neurally evoked responses respectively.**


Dopamine
Adrenaline
Noradrenaline
5HT
Nerve
1000
100
10
1
( Dose ratio -1 )
10
100
1000
Phentolamine (μM)

these values and the corresponding mean values of K shown in table 3.

Regression analyses of the results obtained with phentolamine's inhibition of the 5-HT responses over the range shown in table 3 also gave a slope close to unity (fig 35), however the computed value of K from this regression line was 0·018 $\pm$ S.E.* 0·007 $(\mu M)^{-1}$, this value being close to the mean value given in table 3 and was also significantly different (Student's t test, $P < 0{\cdot}001$) from the other estimates of K given above.

* Estimate of S.E. derived from the t test confidence limits of the regression line (see Colquhoun, 1971).

## DISCUSSION

Whilst direct evidence on the identity of the transmitter released from the nerve terminals of the cockroach salivary gland is lacking it is clear from the present study that the catecholamines adrenaline, noradrenaline and dopamine, are able to mimic closely the effects of the actual transmitter. Dopamine is clearly the most potent of these agonists. It is difficult to separate adrenaline and noradrenaline on the basis of potency but the slope of the log dose-response curve for adrenaline is significantly steeper than those for noradrenaline and dopamine. This suggests that the gland cell receptors are not classical adrenergic receptors, since dopamine has a much less potent agonist action on these than do adrenaline or noradrenaline (see review by Woodruff, 1971). Further evidence to support this conclusion is the failure of certain $\alpha$- and $\beta$ - agonists to produce electrical responses (House et al. 1973). At present it is impossible to differentiate between the dopamine receptors and those for noradrenaline. However, the present results taken together with the previous evidence (Bland et al. 1973; House et al. 1973; Fry et al. 1974) indicate that dopamine, rather than noradrenaline, is likely to be the transmitter at this junction.

The only other biogenic amine tested, apart from 5-HT, was octopamine which turned out to be a very weak agonist; like both noradrenaline and adrenaline it also

has not been detected in this tissue (C.R. House, personal communication).

It is worth noting here that the related amine, N-acetyldopamine, is present in many species of insect (see review by Murdock, 1971), including the cockroach (Mills, Lake & Alworth, 1967) and is known to be the cuticular tanning agent in the blowfly (Karlson & Sekeris, 1962). However this substance fails to change the resting potential or the neurally evoked response in this gland (Ginsborg, House & Turnbull, 1976) and therefore it probably plays no part in neuroglandular transmission in this insect.

Whereas it is not yet possible to distinguish the effects of dopamine from those of noradrenaline it is quite clear from the dose-response studies that the actions of 5-HT are distinct from those of dopamine. These experiments showed that 5-HT could not generate a maximum response as large as the maximum neurally evoked response and in addition the slope of its log dose-response curve was significantly less than that for dopamine. It seems highly unlikely that 5-HT is the transmitter in this tissue because its presence has not been detected in the nerve terminals (Bland et al. 1973). There is evidence, however, that it is the neurohormone for the uninnervated salivary gland of the blowfly (Berridge & Patel, 1968; Berridge, 1973).

The biogenic amines are also capable of eliciting salivary secretion from isolated salivary glands of

N. cinerea (Smith, 1977; House & Smith, 1978), and the dose-dependent characteristics of these responses are similar in several respects to those of the electrical responses. The order of potency for the catecholamines is the same and the maximum responses to these compounds matched that of the neurally evoked maxima. The secretory responses of 5-HT failed to match the maximum secretory response, fell more rapidly than those of the other agonists and the slope of its dose-response curve was significantly less than those for the catecholamines.

On the basis of these similarities it would be tempting to suppose that there are common receptors mediating both sets of responses, however, comparison of the actions of ergometrine and methysergide on the electrical (Ginsborg et al. 1976) and secretory (House & Smith, 1978) responses suggests that there may be a difference (see later).

The evidence against a direct transmitter role for ACh is unequivocal, as is the case for alanine, aspartate, GABA, glycine and glutamate since none of them produces an electrical (Bowser-Riley, 1974; Bowser-Riley & House, 1976) or secretory response (House & Smith, 1978). With regard to the actions of ACh on the electrical response it could be argued that it is perhaps involved in augmenting the release of the transmitter. Certainly it causes spontaneous potentials and also enhances the neurally evoked responses if an anticholinesterase is present. Such a proposed interaction could be thought of as

presynaptic excitation whereby ACh released from one type of nerve terminal potentiates the release of the actual transmitter from another. Hypotheses of cholinergic links in adrenergic transmission have taken several forms (see Kosterlitz & Lees, 1972; Starke, 1977; Westfall, 1977) since that first proposed by Burn & Rand (1959). There is at present no compelling evidence for any of these proposals, including the one mentioned above, although there is at least some evidence for axo-axonal synapses between adrenergic and cholinergic nerves (Ehinger, Falck & Sporrong, 1970). However, at present, knowledge of this aspect of the salivary gland innervation is too fragmentary to allow an attractive analogy to be made. Clearly more experimental evidence is required before a sensible conclusion can be drawn about the role of ACh in this tissue.

The results of this study with phentolamine strongly support the suggestion (Ginsborg et al. 1976) that this compound is a competitive antagonist of the catecholamines and the endogenous transmitter on the post synaptic receptors of the cockroach salivary gland.

This quantitative study of phentolamine's inhibition, based on equation (1), conforms to the classical theory of competitive drug antagonism (Gaddum, 1937; Schild, 1949; Arunlakshana & Schild, 1959), where it is assumed that the agonist and antagonist compete for reversible binding to a single population of receptors and the response is a function only of the fractional occupancy

of the agonist. Based on these assumptions it is predicted that the log-dose response curve for the agonist should be displaced to the right without change in slope and that a plot of log (dose ratio-1) against log antagonist concentration should be linear with unit slope. There are many examples, including the present study, where these predictions have been borne out with great accuracy, for many drugs, over a wide range of concentrations (see Rang, 1971; Furchgott, 1972; Jenkinson, 1973).

It should be pointed out, however, that the quantitative analysis of competitive antagonism may be made within the framework of alternative models for drug receptor activation. These models, which regard the receptor as analogous to an allosteric enzyme, are based on evidence which suggests that some form of cooperative step is involved in the response to certain agonists (see Karlin, 1967; Colquhoun, 1973). The experimental observations to support these models are principally derived from agonist induced conductance changes of the post synaptic membrane, which are assumed to give a direct measure of receptor activation (see Colquhoun, 1975). However, both Karlin (1967) and Colquhoun (1973) have shown that according to these models the predicted form of antagonism is virtually identical to that for the classical theory. At present the available evidence in a number of systems is insufficiently accurate to distinguish between the predictions of the cooperative and

classical theories of competitive antagonism (cf. Colquhoun, 1973, 1975).

Phentolamine is an established $\alpha$-blocker for the catecholamine receptors in certain tissues of the vertebrates. Its affinity constant for the receptors in the cockroach salivary gland is about $1(\mu M)^{-1}$ which is about 100 - 1000 times less than is usually found for the $\alpha$-receptors (Furchgott, 1972) with the exception of its value (ca. 0·5 $(\mu M)^{-1}$ for the $\alpha$-like receptors in the guinea-pig liver (Haylett & Jenkinson, 1973). Other experiments (Ginsborg et al. 1976) have excluded the possibility that the electrical response is mediated by $\alpha$- or $\beta$ receptors in the salivary gland. In addition, the evidence presented so far, albeit indirect, suggests that dopamine is the transmitter at this junction. Therefore it is possible that phentolamine blocks the neurally evoked response by occupying specific dopamine receptors. In this connection it is interesting to note that the affinity constants of the transmitter and dopamine for the receptors mediating the electrical responses are indistinguishable. Phentolamine also blocks dopamine responses in specific neurones in the brain of the snail, Helix aspersa (Walker, Woodruff, Glaizner, Sedden & Kerkut, 1968) where the order of potency for the catecholamines is identical to the agonist induced responses in the acinar cells. From structure-activity studies (Woodruff & Walker, 1969) it was shown that the structural requirements for maximum dopamine-like activity of the snail neurones

were preserved in N-methylated derivatives but lost after O-methylation. These requirements are different from those for maximum $\alpha$- or $\beta$ -agonist activity (see Woodruff, 1971). A similar study by Ginsborg, House & Turnbull (1976) of the effects of methylated derivatives on the acinar cells of the cockroach salivary gland showed the same chemical specificity. Woodruff (1971), from a detailed analysis of the structural requirements for activity on a specific dopamine receptor, predicted that the substance 2-amino-6, 7-dihydroxy-1,2,3,4-tetrahydronapthalene (ADTN) should specifically stimulate dopamine receptors. ADTN has subsequently been found to be active at a number of sites, where dopamine has been postulated as a transmitter (e.g. Woodruff, Elkhawad, Crossman & Walker, 1974; Miller, Horn, Iversen & Pinder, 1974) including the cockroach salivary gland (House & Ginsborg, 1976) where it mimicked the neurally evoked response at concentrations below 1 $\mu$M. These results thus reinforce the supposition that the receptors mediating the neurally evoked hyperpolarizing response in the cockroach salivary gland are specific dopamine receptors.

Although the inhibition of the 5-HT responses by phentolamine may also be competitive it is apparently dissimilar in two respects from that described for the catecholamines and the neurotransmitter. High concentrations were required to inhibit the effects of 5-HT and, moreover, the responses to this agonist fell more

rapidly in the presence of phentolamine. These results suggest a genuine difference between the receptors for 5-HT and those for the catecholamines and perhaps even the operation of different processes of antagonism on them.

The secretory responses of the cockroach salivary gland to nerve stimulation or bath applications of dopamine, noradrenaline, adrenaline and 5-HT are reversibly blocked by phentolamine (House & Smith, 1978). In a quantitative study of this antagonism (Bowser-Riley, House & Smith, 1978) it was found that phentolamine also appeared to inhibit the catecholamine receptors in a competitive manner and the value of the affinity constant for these receptors (ca $1(\mu M)^{-1}$) was found to be identical with those for its inhibition of the electrical response. Higher concentrations were also required for the inhibition of the 5-HT evoked secretory response and, like the electrical response (House et al. 1973), a concentration of phentolamine could be applied to selectively block the secretory responses to nerve and dopamine whilst the 5-HT response remained largely unaffected. In addition the secretory response to 5-HT also declined more rapidly in the presence of this inhibitor which, like the electrical response, was thought to be due to an increase in receptor desensitization (Bowser-Riley et al. 1978). Estimates of K for phentolamine's inhibition of the 5-HT evoked secretory response were 0·08 $(\mu M)^{-1}$ being about five times less than the corresponding value for the electrical response (see table 3). In addition it was found that the

rate of densitization was more marked for the secretory responses than the electrical responses and estimates of K above 50 $\mu$M phentolamine became unreliable and dependent on phentolamine concentration. It was suggested that the increased rate of desensitization of the secretory responses, evoked by long exposure to 5-HT, may account for the disparity in the value of K for the different responses. A similar explanation could be advanced for the unreliable estimates of K for concentrations of phentolamine above 600 $\mu$M for the inhibition of the 5-HT evoked electrical response. If this were the case an enhanced rate of desensitization would account for the observed increases in the equipotent dose ratio for the electrical responses to 5-HT at these concentrations of phentolamine.

However, the results of both the electrical and secretory experiments are consistent with the idea that phentolamine distinguishes between the 5-HT and catecholamine receptors. It is interesting that it also differentiates between similar receptor types in the intestinal muscle of the mollusc Tapes watlingi (Dougan & McLean, 1970). Although 5-HT is not present in detectable amounts in the salivary nerve terminals it is conceivable that this substance is released from distant cells; other possibilities are that the so-called 5-HT receptors normally combine with another transmitter molecule or even that they are redundant.

Although there is a striking resemblance between

certain properties of the receptors mediating the secretory and electrical responses of the cockroach salivary gland, namely their orders of potency, ability to match maximal nerve responses and affinity constants for phentolamine, it seems unlikely that the catecholamine receptors involved in the two types of response are identical. House & Smith (1978) examined the effects on secretion of methysergide and ergometrine which block the electrical responses of the acinar cells to dopamine (Ginsborg et al. 1976). Unlike phentolamine these drugs failed to reduce secretion elicited by dopamine or nerve stimulation. Table 4 shows the effects of these antagonists in other tissues; evidently their success is mixed and ergometrine, in particular has agonist activity.

An explanation of these opposing effects might be made if a more detailed consideration is given to the electrical response. As mentioned earlier the electrical response to nerve stimulation occasionally includes a depolarization following the pronounced hyperpolarizing phase. Similar biphasic responses are also produced somewhat infrequently by iontophoretic application of dopamine (Blackman et al. 1978). Moreover, there is an increase in acinar membrane conductance which outlasts the hyperpolarizing phase of the response (Ginsborg et al. 1974). Taken together these results raise the strong possibility that dopamine may occupy two different receptor types. Where intracellular responses to dopamine have been recorded in molluscan neurones the results suggest

TABLE 4. Properties of some dopamine antagonists.

| Preparation | Dopamine response | Antagonism | Reference |
|---|---|---|---|
| | **Phentolamine** | | |
| Molluscan neurone | Biphasic change in membrane potential | + | Ascher (1972) |
| Molluscan neurone | Hyperpolarization | + | Walker, Woodruff, Glaizner, Sedden & Kerkut (1968). |
| Molluscan intestine | Inhibition of neurally evoked contraction | + | Dougan & McLean (1970) |
| Insect salivary gland | Hyperpolarization | + | Ginsborg et al. (1976) |
| Insect salivary gland | Fluid secretion | + | Bowser-Riley, House & Smith (1978) |
| Cat | Increase in blood pressure | + | van Rossum (1965) |
| Dog | Increase in blood pressure | + | McNay & Goldberg (1966) |
| | **Methysergide** | | |
| Molluscan neurone | Hyperpolarization | + | Woodruff, Walker & Kerkut (1971) |
| Guinea-pig submucous plexus neurone | Hyperpolarization | + | Hirst & Silinsky (1975) |
| Insect salivary gland | Hyperpolarization | + | Ginsborg et al. (1976) |
| Insect salivary gland | Fluid secretion | - | House & Smith (1978) |
| Mouse | Stereotyped locomotor behaviour | - | Milson & Pycock (1976) |
| | **Ergometrine** | | |
| Molluscan neurone | (Hyperpolarization<br>(Depolarization | +*<br>- | Ascher (1972) |
| Molluscan neurone | Hyperpolarization | + | Berry & Cottrell (1975) |
| Molluscan neurone | Hyperpolarization | + | Walker et al. (1968) |
| Insect salivary gland | Hyperpolarization | + | Ginsborg et al. (1976) |
| Insect salivary gland | Fluid secretion | -* | House & Smith (1978) |
| Dog | Renal vasodilatation | + | Bell, Conway & Lang (1974) |
| Rat | Stereotyped locomotor behaviour | -* | Pijnenburg, Woodruff & van Rossum (1973); Woodruff, Elkhawad & Crossman (1974). |

+ Present. - Absent. * Evidence of agonist activity.

at least two kinds of dopamine receptor (Ascher, 1972; Berry & Cottrell, 1975) and in these cases the hyperpolarizing phase of the biphasic dopamine response is selectively blocked by ergometrine. Biphasic responses to nerve stimulation and applied dopamine have been recorded in the cockroach acinar cells and ergometrine seems to be able to selectively suppress the hyperpolarization (Bowser-Riley et al. 1978). In contrast to phentolamine the recovery to ergometrine was slow and incomplete and occasionally led to an enhancement of the secondary depolarization. Selective reduction of the hyperpolarization seems to be achieved by methysergide also (Ginsborg et al. 1976). Thus dopamine may interact with two kinds of receptor, one of which is involved in the hyperpolarization and the other in depolarization and perhaps fluid secretion.

Mutually opposing responses to dopamine are also found <u>in vivo</u>. For example, dopamine infused at high concentrations into cats and dogs produces a rise in blood pressure. This increase masks a transient fall in pressure (Yeh, McNay & Goldberg, 1969), which can be uncovered by selectively inhibiting the rise with $\alpha$-adrenergic antagonists including phentolamine (van Rossum, 1965; McNay & Goldberg, 1966). The fall in pressure has been attributed to activation of specific dopamine receptors in the renal vasculature (van Rossum, 1966; Yeh et al. 1969). This effect can be blocked by ergometrine (Bell et al. 1974) and neuroleptic drugs (Goldberg & Yeh, 1971). These compounds probably come closest to

being specific dopamine antagonists (e.g. Clement-Cormier, Kebabian, Petzold & Greengard, 1974; Miller, Horn & Iversen, 1974) and the cis-isomer of flupenthixol is about the most potent of this group. This substance blocks both the electrical and secretory responses to nerve stimulation and dopamine (House & Ginsborg, 1976; Breward, 1977).

The present quantitative study of the antagonism exerted by phentolamine on the electrical responses in the cockroach salivary gland distinguishes between 5-HT and catecholamine receptors but fails to delineate further between dopamine and noradrenaline, adrenaline or, in fact, the transmitter at this neuroglandular junction. However the receptors are more sensitive to dopamine than noradrenaline and this taken together with other studies (Ginsborg et al. 1976; House & Ginsborg, 1976; Ginsborg, House & Turnbull, 1976) indicates that the neurally evoked electrical response is mediated by specific dopamine receptors. Thus the hypothesis that dopamine is the transmitter at this neuroglandular junction is supported by the present results and may be used as basis for further study.

## GENERAL DISCUSSION

The study of simple invertebrate systems to unravel the complexities of neural mechanisms observed in the higher orders of animals is by no means a recent phenomenon. With particular reference to the cockroach salivary gland Hofer (1887) stated 'One might hope, however, to obtain relevant results by studying invertebrates, where simpler relations might be found'. Whilst it is apparent from the present study that the innervated salivary gland of the cockroach has proved to be a suitable preparation for the study of neuroglandular transmission Hofer's belief in the electrical nature of this process has been shown to be unfounded.

From a variety of experimental approaches not available to the early workers it is now evident that the activity of the cockroach salivary gland is mediated by chemical neuroglandular transmission.

Intracellular recordings from the gland cells show that they respond to nervous stimulation slowly and only after a long latent period (House, 1973; Ginsborg & House, 1976). Although it has not been possible to show directly that no electrical coupling occurs between the nerve terminals and the gland cells these properties are more characteristic of chemical than electrical transmission (see Bennett, 1977).

These electrophysiological inferences as to the mode of transmission would be less satisfactory without the

extensive pharmacological data that have been presented. This shows that there is an identity of action between the response to nerve stimulation and that evoked by the application of known transmitter substances. In addition it has been shown that both types of response are mediated by a common set of post-synaptic receptors. Such properties are well established for chemical transmission where a large variety of agonists and blocking agents are known (cf. Gerschenfeld, 1973; Hubbard, 1973), whereas electrical synapses are generally insensitive to pharmacological agents.

Morphological evidence suggests that the neuro-effector site of the cockroach salivary gland is represented by the numerous axonal swellings found on the acinar nerve plexus and the presence of a chemical mediator associated with them has been demonstrated by both fluorescent (Bland et al. 1973) and ultrastructural techniques (Whitehead, 1971; D.J. Maxwell, personal communication). It has been suggested (see section I) that this feature of the innervation is analogous to the varicosities of the autonomic ground plexus of vertebrates (cf. Hillarp, 1945, 1959; Norberg & Hamberger, 1964; Gabella, 1977) where physiological and histochemical evidence for the <u>en passage</u> release of neurotransmitters has been presented (see Burnstock & Bell, 1974; Geffen & Jarrott, 1977). Although this aspect of the innervation of the cockroach salivary gland has not been investigated the analogy is compelling.

Moreover, certain features of the transmission process in the cockroach salivary gland bear a striking resemblance to those found at the vertebrate autonomic effector sites not only in salivary glands but also in smooth muscle and cardiac muscle cells. For example in salivary gland (Creed & Wilson, 1969; Kagayama & Nishiyama, 1974) smooth muscle (Bolton, 1976) and cardiac muscle cells (Niedergerke & Page, 1977) the responses to nerve stimulation or iontophoretically applied transmitter are characterized by a long latency and duration similar to that found in the cockroach acinar cells by these methods (Ginsborg & House, 1976; Blackman et al. 1978). The nerve-evoked electrical responses in some of these tissues are also graded according to the strength of the stimulus and it has been suggested that the grading is a function of the number of axons activated (Creed & Wilson, 1969; Hirst, 1977). It is difficult to establish the precise innervation of individual cells since like the cockroach salivary gland (Bland & House, 1973; Ginsborg et al. 1974) there is both morphological and physiological evidence to show that the effector cells are electrically coupled (Burnstock, 1970; Bennett, 1972; Garrett, 1974, Hirst, 1978).

The principal point of divergence between the innervation of autonomic end organs and that of the cockroach salivary gland is probably the identity of the transmitter. The salivary glands of vertebrates have a predominantly cholinergic innervation (cf. Garrett, 1966, 1974; Campbell, 1970; Burnstock & Bell, 1974; Kagayama &

Nishiyama, 1974). The other predominant transmitter in the autonomic nervous system is noradrenaline (cf. Norberg & Hamberger, 1964; Gershan, 1970; Krnjevic, 1974; Geffen & Jarrott, 1977). Although this compound is an effective stimulant of cockroach salivary activity, the pharmacological evidence presented indicates that the receptors for the transmitter released from the salivary nerves are not classically adrenergic (see section II). Indeed their properties resemble those of receptors described in other systems where dopamine is likely to be the transmitter (cf. Walker et al. 1968; Woodruff & Walker, 1969; Woodruff, 1971; Miller et al. 1974; Goldberg et al. 1978).

In view of the possible importance of dopamine as a transmitter in the central nervous system of vertebrates (see Hornykiewicz, 1973, 1977; Iversen, 1975; Krnjevic, 1975), the cockroach salivary gland having a peripheral dopaminergic innervation, may provide an extremely useful model for studies on dopaminergic mechanisms at a cellular level.

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For what is left I claim responsibility and certify that it is a product of my own efforts.

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# APPENDIX

Cell Tiss. Res. 187, 525–534 (1978)

Cell and Tissue Research


# The Salivary Glands of the Cockroach *Nauphoeta cinerea* (Olivier)

## A Study of Its Innervation by Light and Scanning Electron Microscopy

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**Summary.** The innervation of the salivary gland of the cockroach *Nauphoeta cinerea* (Olivier) has been investigated with the use of light and scanning electron microscopy. Light microscopy of methylene blue stained glands reveals the presence of a dual innervation arising from the ventral nerve cord and the stomodeal nervous system; the principal innervation is that from the ventral nerve cord which passes to the gland via the reservoir ducts. Branches of these nerves form a plexus on the acinar surface, the axons of which exhibit swelling at irregular intervals. The presence of this surface plexus and the axonal swellings was confirmed by scanning electron microscopy both in normal glands and in those in which the basal lamina had been removed by means of an HCl-collagenase digestion method. No acinar plexus was seen to be formed by branches of the stomatogastric nerve that were associated with the gland. However, other branches of this nerve were clearly connected with a complex network of multipolar neurones on the surfaces of the anterior regions of both salivary reservoirs.

**Key words:** Salivary glands – Insects – Innervation – Light microscopy – Scanning electron microscopy.

## Introduction

It has been suggested that the innervated salivary glands of cockroaches may be suitable organs for studying the mechanisms of neuroglandular transmission

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(House et al., 1973). The innervation of the salivary gland of the cockroach *Nauphoeta cinerea* (Olivier) has been investigated by electrophysiological methods (House, 1973; Ginsborg and House, 1976), but no detailed anatomical study of its innervation has been reported. The gland has a racemose structure, but unlike its counterparts in vertebrates is not enclosed in a fibrous capsule. Thus the surface features of its acini are particularly amenable to investigation by scanning electron microscopy (SEM). The present study was undertaken to provide a detailed account of its innervation to complement the electrophysiological evidence already available and to make comparisons with the innervation of the salivary glands of other species.

## Materials and Methods

All observations were made on the complete salivary apparatus of adult cockroaches *Nauphoeta cinerea*, reared as described previously (House, 1973). Vital staining was achieved by intra-abdominal injection of 0.1–0.2 ml. of 0.5% solution of methylene blue (Dr. G.G. Grübler & Co. Leipzig). At periods between 0.25 and 3 h after injection, the glands were dissected out under a saline of the following composition (mM); NaCl, 160; KCl,1; $CaCl_2$,5; $NaHCO_3$,1; and $NaH_2PO_4$,1; pH 6.9. Tissues were observed on cavity slides either as wet preparations or stored overnight in 5% ammonium molybdate solution, washed in saline, dehydrated in a graded series of ethanols, cleared in xylene and mounted in Xam (Gurr's).

For observation by scanning electron microscopy (SEM) salivary glands were mounted, under saline, on Sylgard (Dow-Corning) filled stainless steel cups and secured by stainless steel pins. All subsequent operations were performed with the gland mounted in this way.

Optimum fixation for SEM was obtained by immersion for one hour at room temperature in 2.5% glutaraldehyde with 8.5% sucrose in 0.05 M phosphate buffer pH 7.2, or 0.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 (modified from Karnovsky, 1965). After fixation the tissue was rinsed several times in phosphate buffer, dehydrated to absolute ethanol, and taken into acetone prior to drying at the critical point of liquid $CO_2$. Tissues were then placed in a vacuum evaporator and coated with gold-palladium before examination on a Cambridge Stereoscan microscope at an accelerating voltage of 10 kV.

HCl-collagenase digestion of the basal lamina was achieved with the method of Evan et al. (1976). Tissues were fixed for three hours, rinsed in phosphate buffer and immersed in 8 N HCl for one hour at 60°C. After HCl digestion tissues were again rinsed and placed in phosphate buffered collagenase (Sigma type II) pH 7.2, at a concentration of 1 mg/ml of buffer, for four hours at 37° C. Tissues were postfixed for 30 min, dehydrated and prepared for SEM as specified above.

**Figs. 1–4.** Light micrographs of methylene blue stained preparations

**Fig. 1.** Salivary duct nerve just posterior to point where secretory ducts fuse with reservoir ducts to form common salivary duct. Nerve (*n*) associated with secretory duct (*Sec. D*) for short distance before crossing to reservoir duct (*Res. D*). Note striated structure of ducts

**Fig. 2.** Salivary duct nerve mid-way down reservoir duct. Note three distinct groups of axons

**Fig. 3.** Group of acini with large branch of duct nerve at right (*n*), forming plexus on acinar surface. Note axonal swellings and at least two axons (*Ax*) traversing surface together

**Fig. 4.** Group of inter-connected densely stained neurones lying on reservoir

**Figs. 5 and 6.** Scanning electron micrographs of normal salivary glands

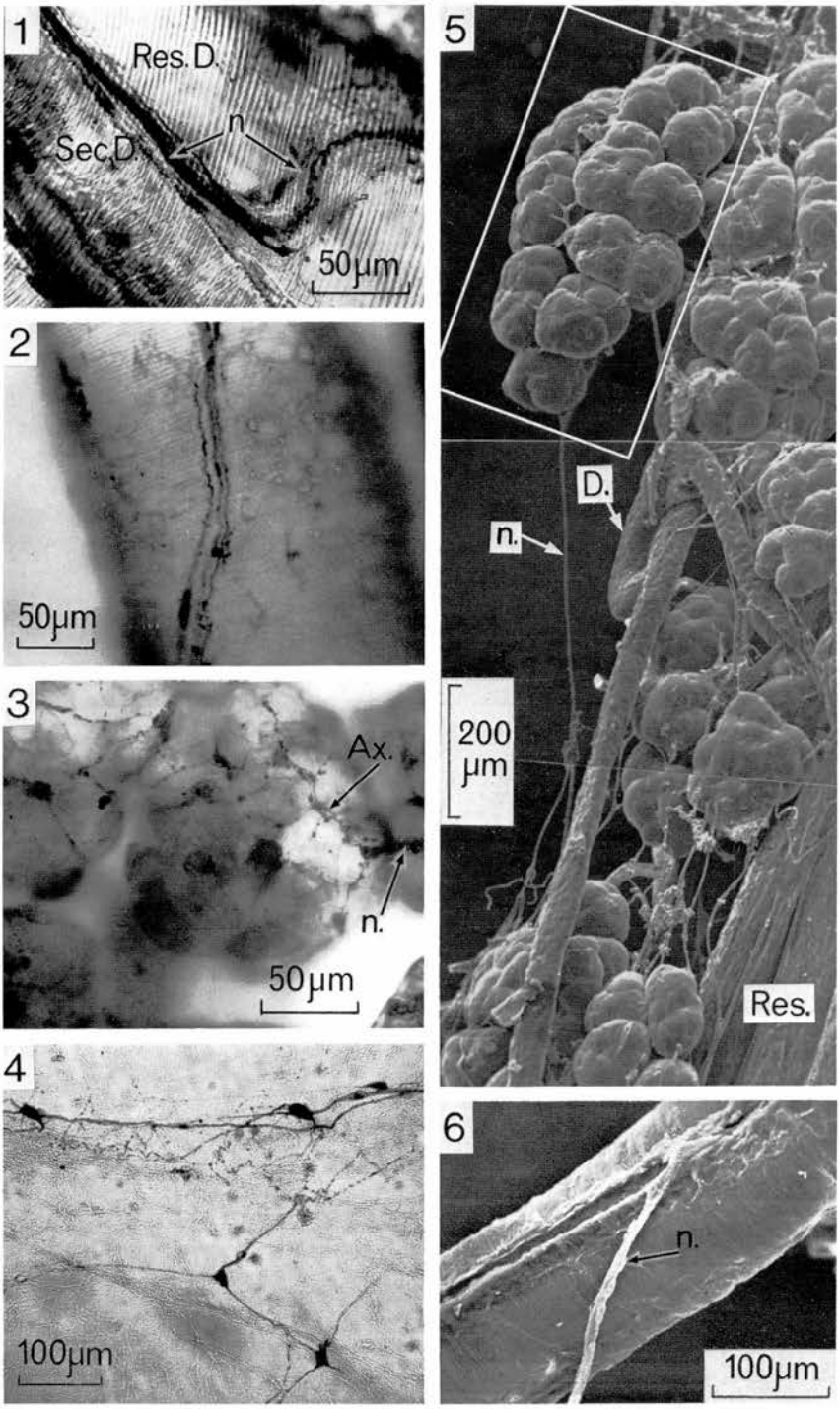



**Fig. 5.** Micrograph montage showing acini (*Ac*), nerves (*n*) secretory ducts (*D*), and part of reservoir (*Res.*)

**Fig. 6.** Nerve trunk (*n*) displaced from connective tissue trough on reservoir duct. Note striations of duct and connective tissue anchors passing around duct

## Results

The paired salivary glands of *Nauphoeta* are composed of acini, ducts and reservoirs, lying between the ventral nerve cord and the crop. The general topography of the gland has been described by Sutherland and Chillseyzn (1968) and the fine structure of the acini and secretory ducts by Bland and House (1971). It receives an innervation from two sources, the ventral nerve cord and the stomatogastric nerve, similar to that described for *Periplaneta* by Whitehead (1971).

Light microscopic examination of methylene blue stained glands showed that a pair of nerves arising from the posteroapical region of the sub-oesophageal ganglion passes on to the gland via the lateral margins of the reservoir ducts (Fig. 1). These duct nerves are composed of several branching axons (Fig. 2), about 3 μm in diameter. The first branch usually passes forward to supply anterior groups of acini, subsequent branches supplying both medial and lateral acinar groups. This is in contrast to *Periplaneta* whose duct nerve is relatively free of branches until it reaches the reservoir mouth (Whitehead, 1971). The lateral acinar groups of *Nauphoeta* are supplied by two or more branches of the ipsilateral duct nerve, whereas central groups receive branches from both the left and right duct nerves. The final branch of the duct nerve supplies the musculature at the mouth of the reservoir; these muscles have been seen to contract following electrical stimulation of the duct nerve (Bowser-Riley, unpublished observations).

Upon reaching the acinar surfaces the nerves undergo profuse branching forming a plexus (Figs. 3, 8) the axons of which exhibit swellings at irregular intervals similar in size to those reported in this and other insect salivary glands (see Table 1). Similar structures have been observed in the vertebrate autonomic nervous system (e.g., Gabella, 1976) and have been called variously swellings, beads, enlargements or varicosities. In the present paper the term axonal swelling will be used.

The stomatogastric nerve is part of the stomodeal nervous system of cockroaches and has been well described by Willey (1961). Principally it innervates the crop, but also gives rise to several fine lateral branches that appear to be

**Table 1.** Axonal swellings in insect salivary glands

| Species | Thickness of axonal swellings | Method | Reference |
|---|---|---|---|
| *Nauphoeta cinerea* | Mean 1.4 μm Range 1.0–3.0 μm | M.B. | Present study |
| | Mean 1.5 μm Range 1.1–1.9 μm | SEM | Present study |
| | Mean 1.9 μm Range 1.1–2.9 μm | FLUOR | Bland et al. (1973) |
| *Periplaneta americana* | Mean 2.1 μm Range 1.1–4.2 μm | M.B. | Whitehead (1971) |
| *Schistocerca gregaria* | Mean 1.8 μm Range 1.0–4.00 μm | FLUOR | Klemm (1972) |

M.B. = methylene blue; SEM = Scanning electron microscopy; FLUOR = technique of Falck for formalin induced fluorescence

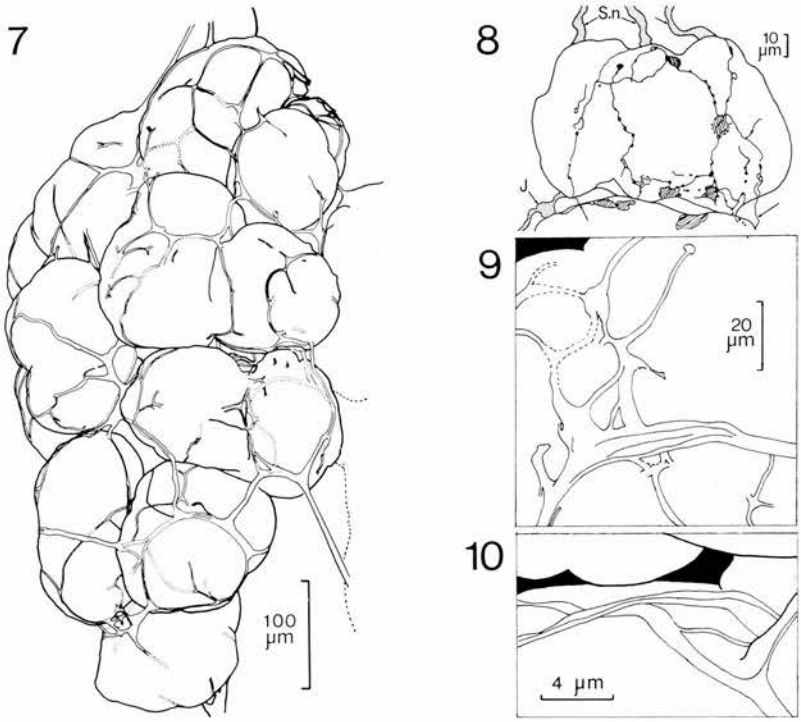



**Fig. 7.** Tracing made from a composite of numerous micrographs at higher magnifications of the group of acini outlined in Figure 5. Note multiple input to this acinar group, extensive surface innervation and nerve associated with secretory duct (lower right). Pathways of acinar axons not established with certainty, because limitations set by geometry of preparation, are indicated by broken lines

**Fig. 8.** Camera lucida reconstruction of methylene blue stained acinus showing extensive network of beaded acinar axons. Note contact with adjacent (lower) acinus (arrowed), input of three sheathed duct nerves (*Sn* and stippled) and complex neural junction (*J* and stippled) off acinar surface. Hatched areas are stained nuclei of acinar cells

**Fig. 9.** Tracing of micrograph in Figure 11, showing course of acinar axons, recourse being made to other micrographs from different angles and magnifications to confirm their presence. Note nerve trunk passing on to acinus showing multiple axons with complex ramifications on acinar surface

**Fig. 10.** Tracing of Figure 12, obtained in same manner as in Figure 9. Note upper axon composed of at least two units

associated with the gland. It proved difficult to follow the more anterior branches of this nerve, since they become enmeshed with the more numerous crop branches and the connective tissue between the gland and the crop. However, some of the posterior branches were traced to acini lying adjacent to the reservoirs, but apparently did not form acinar plexuses similar to those originating from the duct nerve. Other branches of the stomatogastric nerve were found to be continuous with the axons of densely staining multipolar neurones that were confined to the anterior regions of the reservoirs (Fig. 4). These are similar to those reported as sensory neurones in the abdomen of the blowfly larva by Osborne (1963).

Scanning electron micrographs of the gland revealed the same gross structure as that observed with light microscopy, with well-defined acini arranged in groups around the terminations of secretory ducts (Fig. 5). The branching pattern of the ducts was evident only when the gland was viewed from its dorsal surface, i.e., the surface that lies adjacent to the crop. However, glands were usually scanned on the ventral surface to facilitate tracing the acinar axons where they are more numerous.

The duct nerves (Fig. 6) and their branches are enclosed in a substantial sheath which at its junction with the acinar surface becomes continuous with the basal lamina (Fig. 11). A striking feature of the electron micrographs is the richness of the acinar innervation. The acinar axons could be traced as surface ridges under the basement membrane (Figs. 9, 11), forming a similar plexus (Fig. 7) to that observed with light microscopy (Fig. 8). Evidently they traverse the acinar surface for some considerable distance before passing to adjacent acini. At higher magnifications some of these nerves appear as multiple tracts of up to four units (Figs. 11, 12). Although the number of axons could not be estimated with precision it certainly is obvious that acini receive multiple innervation. (Sections of acini observed with transmission electron microscopy also exhibit axon-containing protrusions of the basement membrane of similar dimensions, D.J. Maxwell, personal communication.) Axonal branching occurs not only on the acinar surface (Fig. 11), but also between acini (Fig. 13); in addition acinar axons could occasionally be seen to cross (Figs. 10, 12).

Some of the nerves on the acinar surface exhibit an irregular beaded appearance (Fig. 14) suggesting either axonal convolutions or swellings. To resolve this point it was decided to attempt to strip the basement membrane off the acinar surface by a method used successfully by Evan et al. (1976) on the kidney tubules and autonomic ganglia of the rat. Glands treated in this way maintained their integrity rather well

**Figs. 11–14.** Scanning electron micrographs of normal salivary glands

**Fig. 11.** Complex innervation pattern on acinar surface

**Fig. 12.** Crossing over of acinar axons on surface

**Fig. 13.** Neural branching of acinar nerve between three acini

**Fig. 14.** Axonal swellings (arrows) of acinar axons in normal (unstripped) preparations

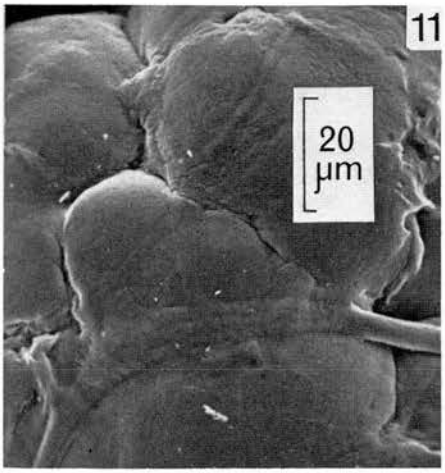
**Figs. 15–18.** Scanning electron micrographs of salivary glands treated with HCl-collagenase to remove basal lamina

**Fig. 15.** Low power view of area of stripped gland. Note clarity of acinar innervation with branch of duct nerve (*n*) passing on to acinar group from reservoir duct (*D*)

**Fig. 16.** Axonal swellings of acinar axon traversing acinar surface

**Fig. 17.** Axon passing between adjacent acini. Note axonal swellings and remains of axonal sheath (*S*). Axonal swellings (arrows) also evident in another surface axon

**Fig. 18.** Fine axon appearing to terminate (arrow) on acinar surface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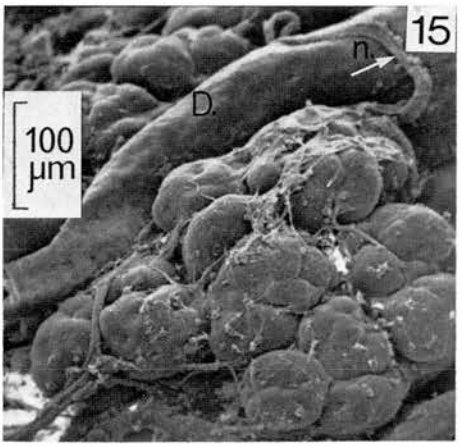
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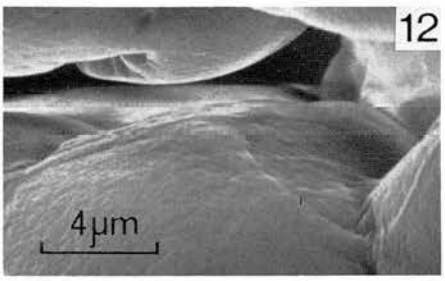
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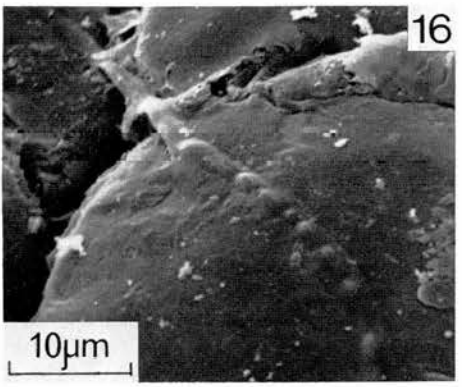
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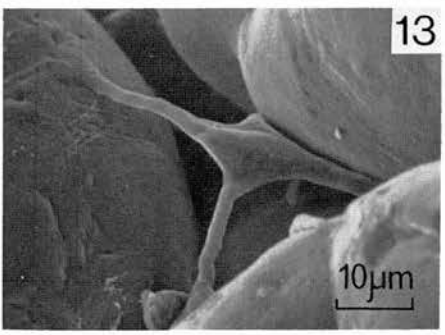
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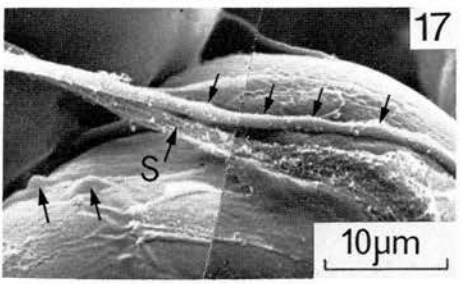
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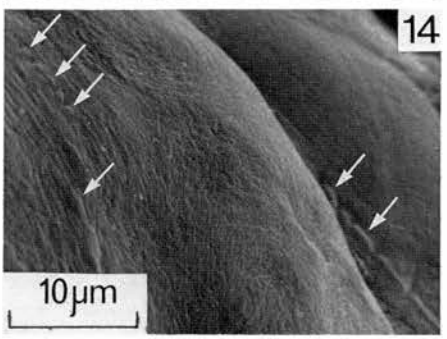
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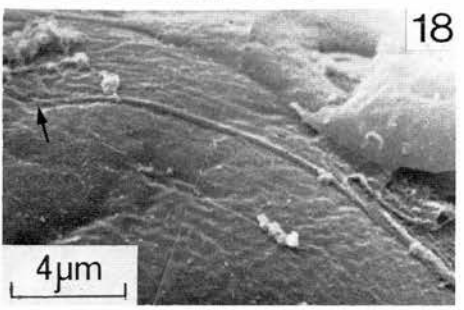
18
4μm

(Fig. 15). Evidence of considerable digestion of the basement membrane could be seen by the presence of debris on the scanned surface not observed in control preparations. In some cases fragments of the basal lamina were removed completely to reveal the underlying structures. Moreover, the outlines of acinar axons could be observed more clearly (Figs. 16, 17). As a result it was possible to examine the acinar surface at higher magnifications and trace fine axons of less than 1 μm (surface dimensions) which apparently terminate on the surface or pass into the acinus (Fig. 18). Furthermore, the beaded appearance of acinar axons seen in unstripped preparations was revealed as due to swellings (Figs. 16, 17), rather than axonal convolutions.

## Discussion

Light microscopy discloses a complex innervation of the salivary gland of the cockroach *Nauphoeta cinerea*. Because of its open structure it has been possible to use the increased resolution and depth of focus inherent in scanning electron microscopy to study the surface innervation.

The principal innervation of the gland arises from the sub-oesophageal ganglion and passes to the gland via the reservoir ducts. A secondary contribution is made by branches of the stomatogastric nerve which is part of the stomodeal nervous system. The salivary glands of the cockroach *Periplaneta americana* (Whitehead, 1971) and the locust *Schistocerca gregaria* (Klemm, 1972) are innervated in a similar manner, whilst those of the moth *Manduca sexta* are innervated solely by the stomatogastric nerve (Robertson, 1974). The duct nerves of *Nauphoeta* have been traced by light and scanning electron microscopy, branching of these nerves occuring as they pass down the reservoir ducts. The difference in the branching pattern of these nerves when compared to those of *Periplaneta* (Whitehead, 1971) may be due to the different topographical relationship in each species, described by Sutherland and Chillseyzn (1968). The number of axons in these nerves is as high as eight when investigated by transmission electron microscopy (D.J. Maxwell, personal communication); a similar number has been observed in *Periplaneta* (Whitehead, 1971). That acinar groups can be supplied by more than one branch of the duct nerve, as observed in the present study, is confirmed by the electrophysiological investigations of acinar innervation by Ginsborg and House (1976). In their experiments on paired salivary glands, in which the duct nerves on each side were separately stimulated, they found that centrally placed acini received at least one axon from each salivary nerve. Salivary secretion from isolated glands of *Nauphoeta* is elicited following stimulation of the duct nerves (Smith and House, 1977), and in *Periplaneta* Whitehead (1970) has observed secretion whilst recording the efferent discharge in the same nerve.

The functional role played by the stomatogastric nerve is unclear. Ginsborg and House (1976) in an electrophysiological study of neuroglandular relationships suggested that the stomatogastric nerve makes no conspicuous contribution and Whitehead (1970) could find no relationship between electrical activity in this nerve and salivary secretion. Nevertheless branches of the stomatogastric nerve, although not so extensive as those of the duct nerves, can be seen to supply the gland in methylene blue stained preparations. Those branches that are associated with acini

may only serve to modify or augment salivary secretion rather than initiate it. Other branches of the stomatogastric nerve have clearly been observed to join the axons of discrete neurones confined to the posterior regions of the reservoirs. These cells, or their ramifying distal processes, may act as stretch receptors that could represent some form of sensory system providing information on pressure changes in the reservoir. Such a function was suggested by Osborne (1963) for similar cells in the abdomen of the blowfly larva.

The extensive plexus formed by branches of the duct nerves bears a close resemblance to those described for other insects (Whitehead, 1971; Klemm, 1972) and vertebrates (Hillarp, 1946; Garrett, 1966). This feature of the innervation is apparently analogous to the autonomic plexus reported by Hillarp (1946, 1959) for a variety of mammalian tissues. As the results of the present study clearly show, any given acinus is traversed by many axons and this probably indicates multiple innervation of cells within the acinus. This concept of multiple innervation was first reported by Lundberg (1955) in the salivary gland of the cat on the basis of electrophysiological criteria. Similar evidence has been presented for *Nauphoeta* by House (1973).

Axonal swellings appear as a consistent feature of acinar axons in this and other insect salivary glands (see Table 1 for methods and references). They also occur with similar dimensions in the terminal portions of the autonomic nervous system of mammals (Hillarp, 1946; Norberg and Hamberger, 1964; see also Gabella, 1976), and have been described as varicosities by these workers. At the ultrastructural level reconstruction of serial sections of the innervation of the iris dilator muscle in the rat (Hökfelt, 1969) and the superior cervical ganglion of the cat (Elfvin, 1963) demonstrates the presence of axonal swellings and in both cases the presence of large numbers of vesicles within them. In the present study, whilst no statement can be made about their contents, axonal swellings can definitely be demonstrated by the HCl-collagenase method in conjunction with SEM of axons both on and off the acinar surfaces. The location of these swellings may represent the two types of neuro-effector sites, interstitial and epithelial, postulated by Garrett (1974) for mammalian salivary glands. Swellings under the basement membrane and on the acinar surface (Fig. 16) represent the epithelial site and those lying off the acinar surface (but still surrounded by basal lamina; Fig. 17) represent the interstitial site. Ultrastructural studies in this laboratory (D.J. Maxwell, personal communication) are not incompatible with this view.

From the evidence available it is clear that the salivary glands of *Nauphoeta* and certain other insects share some common morphological features with those of vertebrates (including a branching ductal tree; discrete acini invested with a nerve plexus; a dual origin of the innervation; axonal swellings in the vicinity of the acinar surface). The most obvious difference is the lack of a connective tissue capsule surrounding the invertebrate gland and this has aided the investigation of the innervation of the cockroach salivary gland considerably.

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# COMPETITIVE ANTAGONISM BY PHENTOLAMINE OF RESPONSES TO BIOGENIC AMINES AND THE TRANSMITTER AT A NEUROGLANDULAR JUNCTION

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SUMMARY

1. A quantitative study has been made of phentolamine's inhibition of the electrical and secretory responses of the isolated salivary glands of *Nauphoeta cinerea* Olivier to nerve stimulation and bath applications of agonists.

2. The results suggested that phentolamine is a competitive antagonist having an affinity constant of about 1 $\mu M^{-1}$ for the receptors for dopamine, noradrenaline, adrenaline and the neurotransmitter.

3. Phentolamine also inhibited responses to 5-hydroxytryptamine in a manner seemingly more complex than competitive antagonism. Attempts to estimate the affinity constant gave values of about 0·08 and 0·015 $\mu M^{-1}$ for inhibition of the secretory and electrical responses respectively.

4. This investigation showed that phentolamine discriminates between two kinds of receptor in this gland, one binding 5-hydroxtryptamine and the other combining with catecholamines and the neurotransmitter.

## INTRODUCTION

Salivary secretion in some insects is apparently under nervous control. The isolated glands of the cockroach copiously produce fluid in response to nerve stimulation or ambient dopamine (Smith & House, 1977). Electrophysiological methods indicate that dopamine and the neurotransmitter elicit similar changes in the membrane potential and conductance of the acinar cells (Ginsborg, House & Silinsky, 1974). Both the electrical and secretory responses to dopamine are dose-dependent (Bowser-Riley & House, 1976; House & Smith, 1978) and can be suppressed by phentolamine, an $\alpha$-adrenergic antagonist (Ginsborg, House & Silinsky, 1976; House & Smith, 1978).

A quantitative study of phentolamine's blocking action has been undertaken to establish whether this antagonist can distinguish between the receptors for the neurotransmitter and dopamine. Our approach was set perforce within a classical framework (e.g. Schild, 1949; Stephenson, 1956) because ignorance of the events associated with receptor activation in the gland cells excludes the use of other models (*cf.* Colquhoun, 1975). Preliminary accounts of parts of this study have been read as communications to the Biochemical and Physiological Societies (Smith, 1977).

## METHODS

The preparation and procedures for the secretory experiments were as described previously (House & Smith, 1978).

In other experiments glands were mounted in a Perspex chamber (volume 4·5 ml.) and intracellular recordings from acinar cells were made as described by Ginsborg *et al.* (1974). Glass micro-electrodes filled with 3 M-KCl having resistances of 20–40 MΩ were used. Membrane potentials were displayed on a Tektronix oscilloscope 502A and recorded with a pen recorder (Devices M2) or oscilloscope camera (Nihon Kohden Kogyo Co. Ltd). Electrical stimuli were delivered to a suction electrode holding the salivary duct nerves. The stimuli (10–60V) were rectangular pulses (0·5 msec duration) from an isolated stimulator (Devices Ltd) and were given in short trains of 1–200 stimuli at 100 Hz. The interval between successive trains was usually 2–3 min.

Solutions of agonists, namely dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine, were applied to glands and the ensuing secretory and electrical responses recorded. Normally the flow rate through the experimental chambers was 2 ml./min and it was increased to about 20 ml./min for perfusion of the agonist solutions. When solutions containing phentolamine were applied to the preparation these were perfused initially at 20 ml./min for about 2 min to ensure rapid mixing in the chambers and thereafter at 2 ml./min. The inhibition of agonist responses by phentolamine was not examined until this antagonist had been present in the chamber for at least 15 min when its blocking action would have reached a maximum (Ginsborg *et al.* 1976; House & Smith, 1978). All drug solutions were prepared as described by House & Smith (1978).

## RESULTS

### *Secretory responses*

It has been shown (House & Smith, 1978) that a pronounced rise in fluid secretion from isolated salivary glands is caused by nerve stimulation which can be imitated in this respect by bath applications of dopamine, noradrenaline, adrenaline or 5-hydroxytryptamine (5-HT). Phentolamine reversibly inhibits these responses and the reduction it produces can be cancelled by increasing the number of nerve stimuli or the agonist concentration. This is illustrated in Fig. 1*A* and *B* where the inhibition was overcome by a fourfold increase in nerve stimuli and a tenfold rise in the concentration of noradrenaline. Since the suppression can be surmounted in that manner it suggests that phentolamine combines with post-junctional receptors for the neurotransmitter and these agonists.

The action of phentolamine was studied by monitoring its effects on the dose–response relationship for each agonist. Dose–response curves were constructed as previously described (House & Smith, 1978); the maximal secretory rate or total secretory volume recorded during a 10 min exposure to an agonist was plotted against the logarithm of its concentration. A typical example is shown in Fig. 1*C* where phentolamine caused a parallel shift of the log dose–response curve for noradrenaline. Other experiments indicated that the parallel displacement was proportional to the phentolamine concentration. Attempts to match maximal responses were successful for all agonists except 5-HT (see later).

Our results suggest that there is competitive antagonism between phentolamine and the catecholamines. Thus it should be possible to estimate the affinity constant $K$ of this inhibitor for the receptors by obtaining matching responses before and during its application. According to Schild (1949) the equipotent dose-ratio, $x$, is given by

$$x - 1 = K\,[\mathrm{I}], \qquad (1)$$

where [I] is the concentration of the antagonist. The method of equal responses, i.e. a *null experiment*, avoids any assumption about the relation between the amplitude of the response and the fraction of receptors occupied and thus allows the validity of equation (1) to be examined. Null experiments, however, generally yield results which are also virtually identical to the predictions of various co-operative models (see Colquhoun, 1975). Nevertheless, at present it seems more appropriate to our study to adopt the simpler classical view of antagonism.

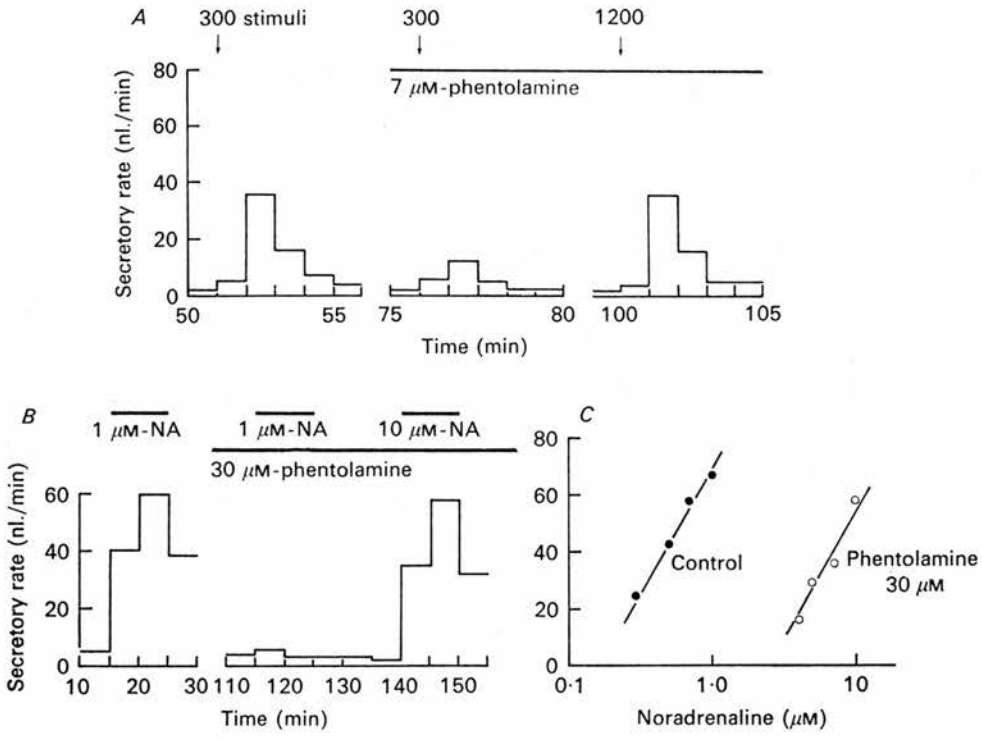


Fig. 1. Effect of phentolamine on secretory responses to nerve stimulation and noradrenaline (NA). *A*, nerve stimuli (arrows) were delivered at 10 Hz. Suppression of neurally evoked response was surmounted by increasing number of stimuli. *B*, responses to bath applications of noradrenaline before and during exposure to 30 μM phentolamine. The amplitudes of these responses and others from the same experiment were plotted as log dose–response curves in *C*. Responses in *A* were obtained in a different experiment from those illustrated in *B* and *C*. In this and all subsequent figures the durations of exposure to drugs are indicated by horizontal bars.

The results illustrated in Fig. 1*C* permit one to calculate an affinity constant for phentolamine which in this case was applied at 30 μM. The dose–response curves were parallel and the equipotent dose-ratio was 16 giving an affinity constant of 0·5 $\mu M^{-1}$. Similar experiments were made for all the agonists. For the catecholamines the values of $K$ were the same, but much smaller estimates were obtained with 5-HT as agonist (Table 1). This difference was confirmed in three additional experiments where 5-HT and dopamine were applied to the same preparations. In the example illustrated in Fig. 2 the 5-HT response was bracketed by dopamine responses, the

TABLE 1. Estimates of affinity constants from experiments on phentolamine's inhibition of secretory responses

| Phentolamine concn. ($\mu$M) | Equipotent dose-ratio | Affinity constant ($\mu$M$^{-1}$) |
|---|---|---|
| | Dopamine | |
| 3 | 3·3 | 0·77 |
| 10 | 8·0 | 0·70 |
| 50 | 44 | 0·86 |
| 100 | 97 | 0·96 |
| 500 | 480 | 0·96 |
| | mean ± S.E. | 0·85 ± 0·05 |
| | Noradrenaline | |
| 3 | 3·2 | 0·73 |
| 10 | 13 | 1·2 |
| 30 | 16 | 0·50 |
| 50 | 48 | 0·94 |
| 100 | 32 | 0·31 |
| | mean ± S.E. | 0·74 ± 0·16 |
| | Adrenaline | |
| 3 | 2·2 | 0·40 |
| 10 | 5·5 | 0·45 |
| 30 | 11 | 0·33 |
| 100 | 170 | 1·7 |
| 500 | 480 | 0·96 |
| | mean ± S.E. | 0·77 ± 0·26 |
| | 5-hydroxytryptamine | |
| 10 | 1·4 | 0·040 |
| 20 | 2·8 | 0·090 |
| 25 | 2·8 | 0·072 |
| 30 | 5·5 | 0·15 |
| 40 | 2·6 | 0·040 |
| | mean ± S.E. | 0·08 ± 0·02† |

† Significantly different from other means ($P < 0·001$).

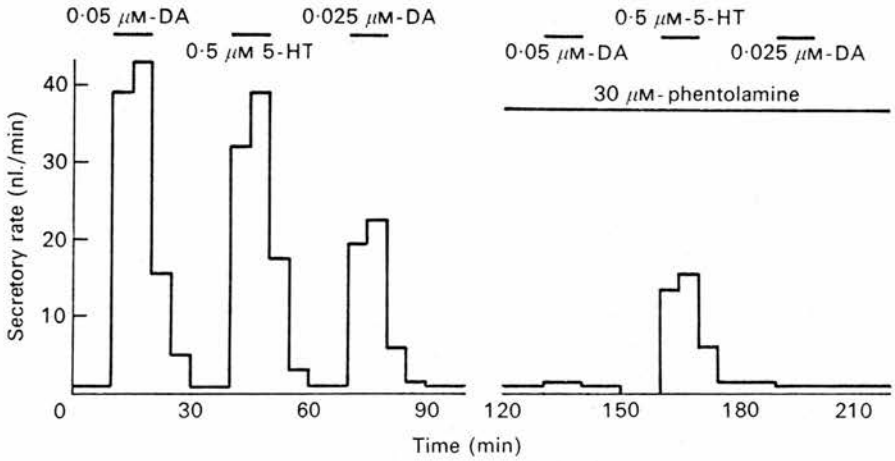


Fig. 2. Effect of phentolamine on responses to dopamine (DA) and 5-hydroxytryptamine (5-HT). In the presence of 30 $\mu$M-phentolamine the bracketing responses to dopamine were reduced more effectively than that to 5-HT.

values of each lying on the linear regions of their respective dose–response curves. On the basis of our measurements of $K$ it was possible to choose a suitable phentolamine concentration which blocked the dopamine responses without abolishing the

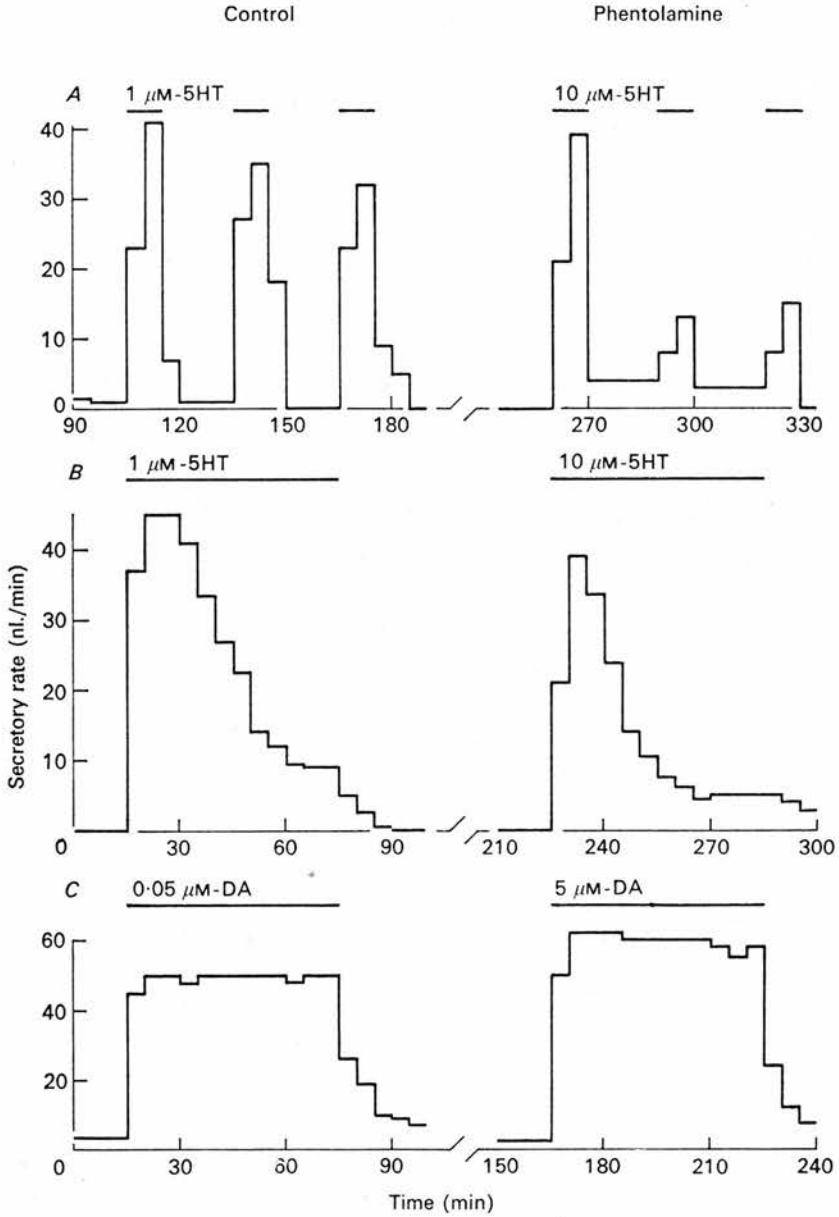


Fig. 3. Action of phentolamine on time courses of responses to 5-HT and dopamine. *A*, responses to the same concentration of 5-HT declined more rapidly in the presence of phentolamine. To achieve matched responses the 5-HT concentration was increased tenfold during application of phentolamine. *B*, prolonged exposures to 5-HT showed a similar effect to that seen in *A*. In *C* the time course of the response to a prolonged exposure to dopamine was unaffected by phentolamine. Responses in *A*, *B* and *C* were recorded in experiments on different preparations.

5-HT response (Fig. 2). In the remainder of the experiment (not shown) the suppression of these responses was overcome by a sixtyfold increase in the dopamine concentrations and a fivefold rise in that of 5-HT. These results strongly suggest that the

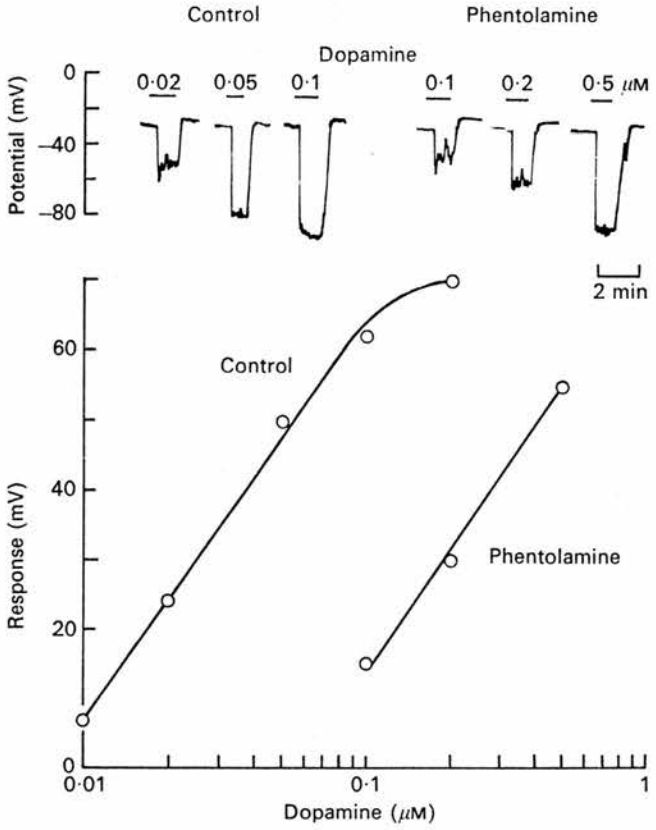


Fig. 4. Antagonism by phentolamine of the electrical response of an acinar cell to dopamine. Upper part shows some intracellular recordings of hyperpolarizing responses to dopamine before and during application of 20 μM-phentolamine. The experimental results are plotted as log dose–response curves in the lower part of the Figure.

TABLE 2. Estimates of affinity constants from experiments on phentolamine's inhibition of electrical responses

| Phentolamine concn. (μM) | Equipotent dose-ratio | Affinity constant ($\mu M^{-1}$) |
|---|---|---|
| | Dopamine | |
| 2 | 3·0 | 1·0 |
| 5 | 6·0 | 1·0 |
| 10 | 10 | 0·90 |
| 20 | 8·0 | 0·35 |
| 30 | 130 | 4·3 |
| 30 | 100 | 3·3 |
| 60 | 64 | 1·1 |
| 100 | 70 | 0·69 |
| 500 | 500 | 1·0 |
| | mean ± S.E. | 1·5 ± 0·4 |

TABLE 2 (*cont.*)

| Phentolamine concn. ($\mu$M) | Equipotent dose-ratio | Affinity constant ($\mu$M$^{-1}$) |
|---|---|---|
| | Noradrenaline | |
| 2 | 3·3 | 1·2* |
| 5 | 8·3 | 1·5 |
| 10 | 7·4 | 0·64 |
| 10 | 10 | 0·90 |
| 30 | 25 | 0·80* |
| 50 | 150 | 3·0 |
| 100 | 300 | 3·0 |
| 200 | 260 | 1·3* |
| | mean ± S.E. | 1·5 ± 0·3 |
| | Adrenaline | |
| 3 | 3·5 | 0·83 |
| 10 | 9·5 | 0·85* |
| 30 | 28 | 0·90* |
| 100 | 120 | 1·2* |
| 100 | 90 | 0·89 |
| | mean ± S.E. | 0·93 ± 0·07 |
| | Nerve | |
| 1 | 1·7 | 0·70 |
| 1 | 1·7 | 0·70 |
| 1 | 1·6 | 0·60 |
| 2 | 4·3 | 1·7 |
| 3 | 7·0 | 2·0 |
| 3 | 2·1 | 0·37 |
| 5 | 2·5 | 0·30 |
| 5 | 4·3 | 0·66 |
| 5 | 6·7 | 1·1 |
| 5 | 4·5 | 0·70 |
| 7 | 4·5 | 0·50 |
| 10 | 5·5 | 0·45 |
| 10 | 15 | 1·4 |
| 30 | 50 | 1·6 |
| | mean ± S.E. | 0·91 ± 0·14 |
| | 5-hydroxytryptamine | |
| 50 | 2·0 | 0·020 |
| 100 | 2·9 | 0·019 |
| 200 | 3·0 | 0·010 |
| 500 | 5·2 | 0·008 |
| 500 | 10 | 0·018 |
| 600 | 12 | 0·018 |
| 600 | 8·2 | 0·012 |
| | mean ± S.E. | 0·015 ± 0·002† |

* Obtained in the same cell for each agonist.

† Significantly different from other means ($P \leqslant 0·01$).

receptors binding 5-HT are different from those combining with the catecholamines.

Table 1 shows that the estimates of $K$ for the catecholamine receptors were independent of phentolamine concentration over a wide range (3–500 $\mu$M). The suppression of the 5-HT response was not so straightforward. At concentrations of

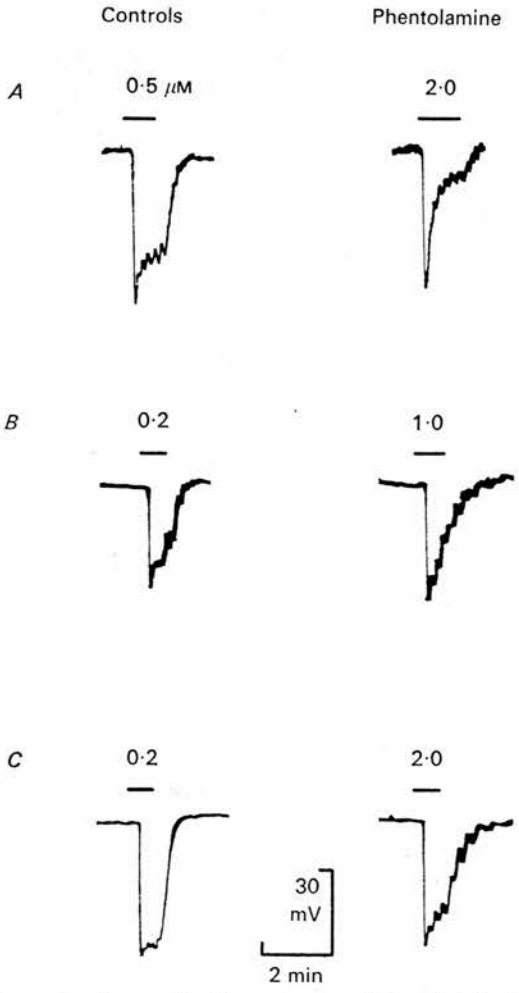


Fig. 5. Action of phentolamine on the time course of the electrical responses of acinar cells to 5-HT. Three representative intracellular recordings from different preparations are shown in $A$, $B$ and $C$ where the resting potentials were $-40$, $-34$ and $-36$ mV respectively. The 5-HT concentrations applied are indicated above the horizontal (duration) bars. The phentolamine concentrations were 200, 500 and 600 $\mu$M in $A$, $B$ and $C$ respectively and in each case caused a more rapid fall in the response than that observed in the corresponding controls.

antagonist above 50 $\mu$M the maximal response could not be matched no matter how large the 5-HT concentration was made. Moreover, reliable estimates of $K$ could not be found because the responses progressively declined in size. A similar but slower fall was observed in the absence of the inhibitor by House & Smith (1978)

who tentatively ascribed the decline to desensitization of the 5-HT receptors. The possibility that phentolamine accelerates the rate of desensitization or some other process having similar effects was examined (Fig. 3). When 5-HT was applied either as brief or prolonged doses the magnitude of the responses fell more rapidly in the presence of the antagonist. For example, the tails of the responses in Fig. 3*B* are exponential with time constants of 20 and 13 min. In contrast, the dopamine response did not fall faster when phentolamine was present (Fig. 3*C*).

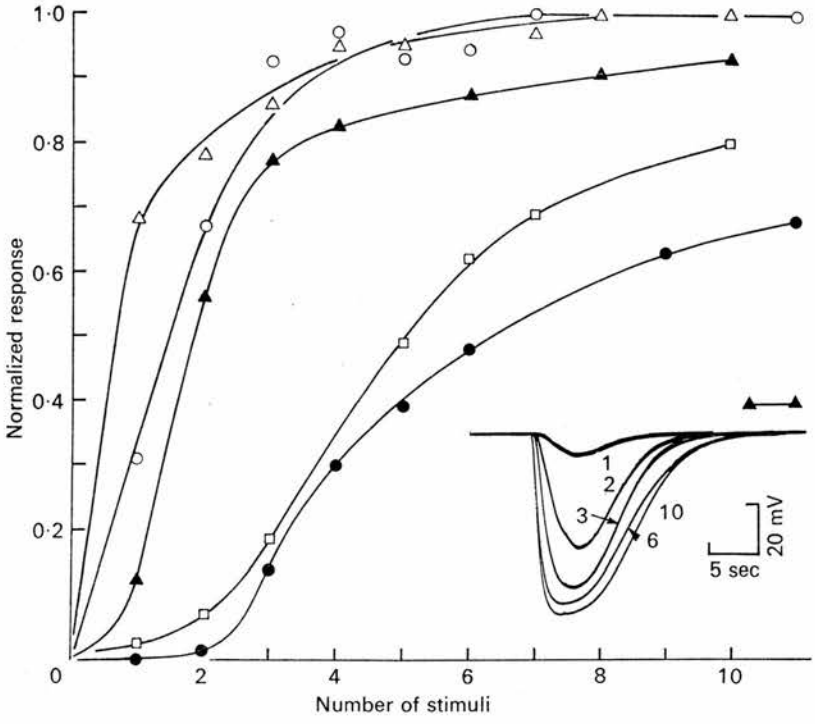


Fig. 6. Typical stimulus–response curves for neurally-evoked hyperpolarizing responses in acinar cells. Each symbol denotes a separate experiment on a different preparation. Some representative intracellular recordings of responses from one of these cells are superimposed in the inset, the number of nerve stimuli being placed beside the corresponding response. The resting potential of this cell was −32 mV and its stimulus–response curve is indicated by ▲. For each cell responses were normalized by dividing them by the corresponding maximal response.

*Electrical responses*

The electrical response of the acinar cells to nerve stimulation comprises a hyperpolarization occasionally followed by a relatively slow and small depolarization. Evidently these opposite phases are independent processes activated by different receptors (Ginsborg & House, 1976). Although different neurotransmitters may be involved it is not obligatory since ionophoretic application of dopamine can also produce a biphasic response (J. G. Blackman, B. L. Ginsborg & C. R. House, unpublished). The following experiments concern the antagonism of the hyperpolarizing response to nerve stimulation or bath-applied agonists.

*Agonist response.* Phentolamine reversibly blocks the hyperpolarization evoked

by dopamine, noradrenaline or adrenaline in a competitive manner. The results of a typical experiment are shown in Fig. 4. Intracellular recordings are illustrated above the corresponding log dose–response curves. In this example the phentolamine concentration was 20 $\mu$M and the equipotent dose-ratio was 8; hence the affinity constant was calculated as 0·35 $\mu M^{-1}$. Similar experiments on the responses to noradrenaline, adrenaline and 5-HT gave the estimates of $K$ shown in Table 2.

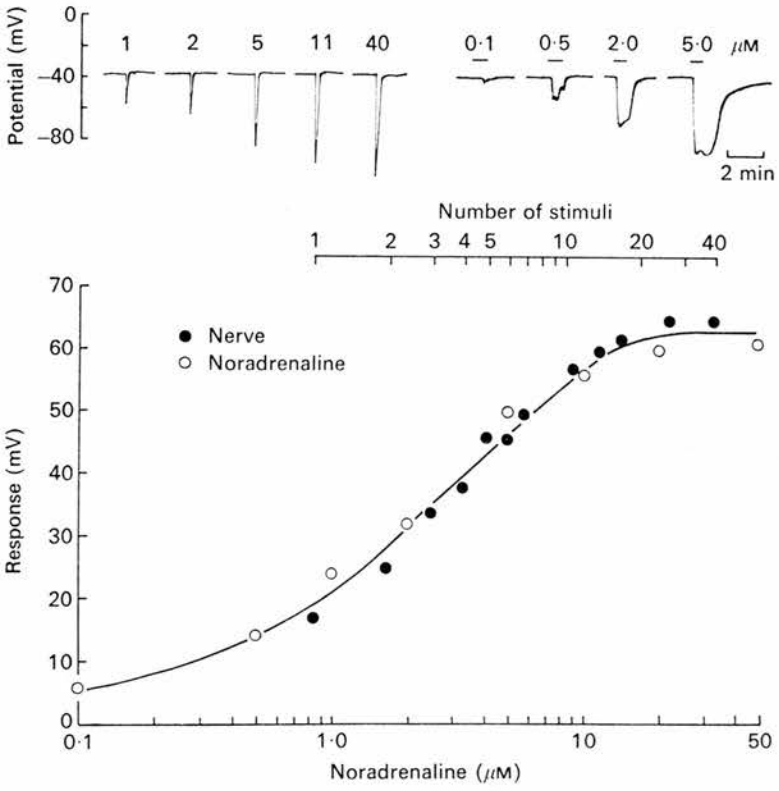


Fig. 7. Stimulus–response relationships of an acinar cell for the hyperpolarizing responses to nerve stimulation and noradrenaline application. Intracellular recordings of some of these responses are shown above the corresponding log dose–response (○) and log stimulus number–response (●) curves. These curves have been superimposed by an appropriate positioning of the abscissae. The number of nerve stimuli and noradrenaline concentrations are given above the responses which have been extracted from a continuous record of membrane potential.

There was no disparity between the affinity constants obtained in the experiments with the different catecholamines nor was there an evident relation between $K$ and the phentolamine concentration. However, phentolamine's affinity constant for the 5-HT receptors was about 100 times smaller than the other estimates. High concentrations of phentolamine were required for suppression of the 5-HT response and yet $K$ was apparently independent of concentration over a tenfold range. Above 600 $\mu$M, however, estimates of $K$ exceeded the values in Table 2 and increased proportionately with inhibitor concentration. It was also observed with high concentrations of phentolamine that the response to 5-HT fell more rapidly (Fig. 5). This

phenomenon resembles the effect of the antagonist on the secretory response (Fig. 3) and may arise from an acceleration of receptor desensitization.

*Neurally evoked response.* It was of particular interest to determine whether the method of equal responses could be applied to the inhibition of transmitter action. Previous evidence (Bowser-Riley & House, 1976) that the amplitude of the hyperpolarization is graded with the number of nerve stimuli suggested that a null experiment might be possible. The relationships between amplitude and number of stimuli are shown in Fig. 6 for several cells, the inset illustrating some intracellular recordings

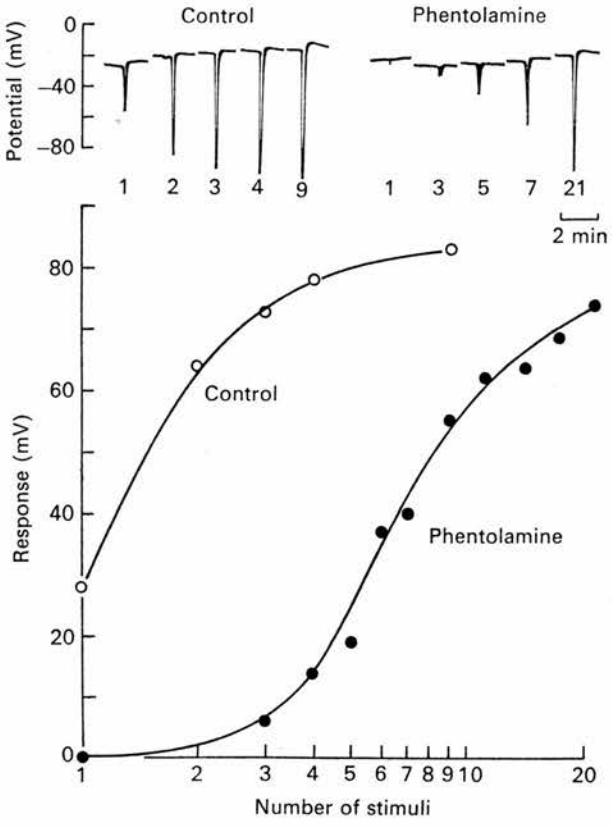


Fig. 8. Antagonism by phentolamine of the hyperpolarizing response of an acinar cell to nerve stimulation. Intracellular recordings of some responses are illustrated in the upper part, the number of nerve stimuli being given below each response. The corresponding log stimulus number–response curves before and during the presence of 10 $\mu$M-phentolamine are shown also.

from one of them. The stimulus–response relation for this cell had an inflexion near the origin and similar changes in slope were found in other cases where a single stimulus produced no response or a small one (*cf.* Ginsborg & House, 1976). The stimulus–response curves for numerous cells, however, were rectangular hyperbolas and resembled agonist dose–response curves when replotted against the logarithm of the number of stimuli. An example is illustrated in Fig. 7 where the dose–response curves for nerve and noradrenaline stimulation have been superimposed. Accurate

superposition was also possible for nerve and dopamine stimulation, but not for 5-HT because the maximal response to this agonist was always less than the maximum to nerve stimulation and the slope of its dose–response curve was too shallow (*cf.* Table 1, Bowser-Riley & House, 1976).

It is not too far-fetched to believe that the local concentration of transmitter at the neuroglandular junction is proportional to the number of stimuli over a modest range. We have taken this as a working hypothesis to explain the similarity between the response curves (e.g. Fig. 7) and have explored its consequences concerning phentolamine's action. The equipotent dose-ratio for transmitter has been estimated as the ratio of stimulus numbers required for equal responses. A typical experiment is illustrated in Fig. 8; intracellular recordings are shown above the stimulus–response curves. Evidently phentolamine caused a parallel shift of the curve, the equipotent number ratio being about 5. Since the concentration of phentolamine was 10 $\mu$M the affinity constant was calculated as 0·4 $\mu M^{-1}$. Thirteen similar experiments were made at different inhibitor concentrations (1–30 $\mu$M). These gave values of $K$ in the range 0·3–2·0 $\mu M^{-1}$ (see Table 2). It was not possible to do satisfactory experiments at concentrations above 30 $\mu$M since the maximal response could not be matched even with large stimulus numbers ($> 500$). It was considered, however, that transmitter output per stimulus would decline towards the end of such trains and hence invalidate the main assumption of our working hypothesis. Indeed there is evidence for a large fall in noradrenaline release from vertebrate sympathetic fibres as the number of stimuli approaches 1000 (Stjärne, Hedquist & Bygdeman, 1969). In view of that limitation an essential condition for successful experiments was that a single stimulus should evoke a large response.

## DISCUSSION

The results of the present study strongly support the suggestion (Ginsborg *et al.* 1976) that phentolamine is a competitive antagonist of dopamine in the cockroach salivary gland. According to eqn. (1) one would expect a linear relation with unit slope between log $(x-1)$ and log $[I]$. It was not feasible to test this relation in most preparations because of the long experimental period required. In two experiments on the electrical response, however, several concentrations of phentolamine were applied to the same glands. The values of $K$ obtained (Table 2) were independent of concentration and this reflected the general agreement with the classical theory of competitive antagonism which is apparent in our results (Fig. 9). Regression analyses of the results of electrical and secretory experiments (left and right series in Fig. 9) confirmed that the slopes were close to unity for the catecholamines and the neurotransmitter.

The suppression of the 5-HT response by phentolamine appears dissimilar in two respects from that described for the catecholamines. High concentrations were required to inhibit the effects of 5-HT and, moreover, the responses to this agonist fell more rapidly in the presence of phentolamine. These results suggest a genuine difference between the receptors for 5-HT and those for catecholamines and perhaps even the operation of different processes of antagonism on them. It is possible that phentolamine enhances the rate of desensitization of 5-HT receptors. Given the

additional presence of such a process reducing the amplitude of the inhibited response it is not surprising that secretion (evoked by a long exposure to the agonist) was blocked at lower phentolamine concentrations than the electrical response. This

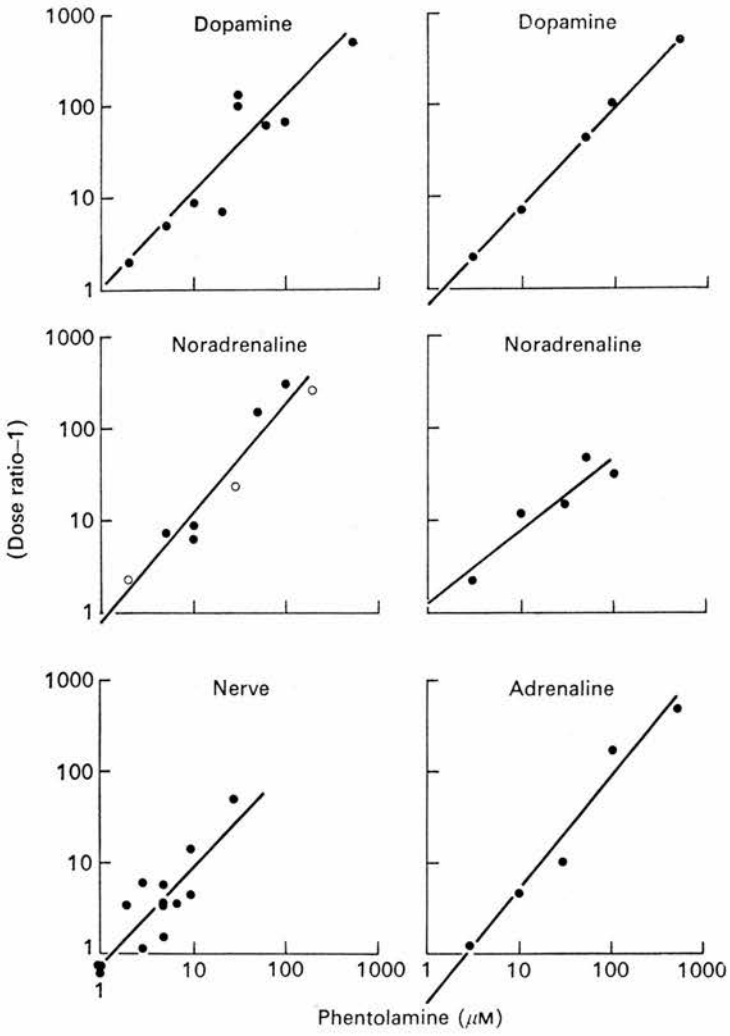


Fig. 9. Analysis of phentolamine's inhibition of electrical and secretory responses. The left hand series show combined results from experiments on the hyperpolarizing responses to nerve stimulation and the agonists indicated whereas the right-hand series refer to the corresponding secretory experiments. Each symbol denotes the result of a single experiment except for ○ indicating three measurements on the same cell. The plots have been made to test the validity of eqn. (1), namely $x - 1 = K[I]$, where $x$ = equipotent dose-ration and $[I]$ = phentolamine concentration. The lines are computed regressions from the data and the slope of each is close to unity as predicted by (1). The computed slopes (± S.E.) for electrical responses to dopamine, noradrenaline and nerve stimulation are $1{\cdot}01 \pm 0{\cdot}18$, $1{\cdot}14 \pm 0{\cdot}14$, $1{\cdot}09 \pm 0{\cdot}17$ and for secretory responses to dopamine, noradrenaline and adrenaline $1{\cdot}06 \pm 0{\cdot}02$, $0{\cdot}79 \pm 0{\cdot}19$ and $1{\cdot}24 \pm 0{\cdot}14$. The values for adrenaline-evoked electrical responses (not shown) are $1{\cdot}06 \pm 0{\cdot}04$ (five measurements).

may account for the disparity between the values of $K$ for the different responses to 5-HT (see Tables 1 and 2).

Our results are consistent with the notion that phentolamine distinguishes between 5-HT and catecholamine receptors. It is interesting that it also differentiates between

TABLE 3. Properties of some dopamine antagonists

| Preparation | Dopamine response | Antagonism | Reference |
|---|---|---|---|
| | *Phentolamine* | | |
| Molluscan neurone | Biphasic change in membrane potential | + | Ascher (1972) |
| Molluscan neurone | Hyperpolarization | + | Walker, Woodruff, Glaizner, Sedden & Kerkut (1968) |
| Molluscan intestine | Inhibition of neurally evoked contraction | + | Dougan & McLean (1970) |
| Insect salivary gland | Hyperpolarization | + | Ginsborg *et al.* (1976) |
| Insect salivary gland | Fluid secretion | + | Present paper |
| Cat | Increase in blood pressure | + | van Rossum (1965) |
| Dog | Increase in blood pressure | + | McNay & Goldberg (1966) |
| | *Methysergide* | | |
| Molluscan neurone | Hyperpolarization | + | Woodruff, Walker & Kerkut (1971) |
| Guinea-pig submucous plexus neurone | Hyperpolarization | + | Hirst & Silinsky (1975) |
| Insect salivary gland | Hyperpolarization | + | Ginsborg *et al.* (1976) |
| Insect salivary gland | Fluid secretion | − | House & Smith (1978) |
| Mouse | Stereotyped locomotor behaviour | − | Milson & Pycock (1976) |
| | *Ergometrine* | | |
| Molluscan neurone | Hyperpolarization<br>Depolarization | +*<br>− | Ascher (1972) |
| Molluscan neurone | Hyperpolarization | + | Berry & Cottrell (1975) |
| Molluscan neurone | Hyperpolarization | + | Walker *et al.* (1968) |
| Insect salivary gland | Hyperpolarization | + | Ginsborg *et al.* (1976) |
| Insect salivary gland | Fluid secretion | −* | House & Smith (1978) |
| Dog | Renal vasodilatation | + | Bell, Conway & Lang (1974) |
| Rat | Stereotyped locomotor behaviour | −* | Pijnenburg, Woodruff & van Rossum (1973); Woodruff, Elkhawad & Crossman (1974) |

+ Present. − Absent. * Evidence of agonist activity.

similar receptor types in intestinal muscle (Dougan & McLean, 1970). The nerve terminals in the cockroach salivary gland contain a catecholamine (Bland, House, Ginsborg & Laszlo, 1973) whereas 5-HT is not present in detectable amounts. Although it is conceivable that this substance is released from distant cells, another possibility is that the so-called 5-HT receptors normally combine with another transmitter molecule or even that they are redundant.

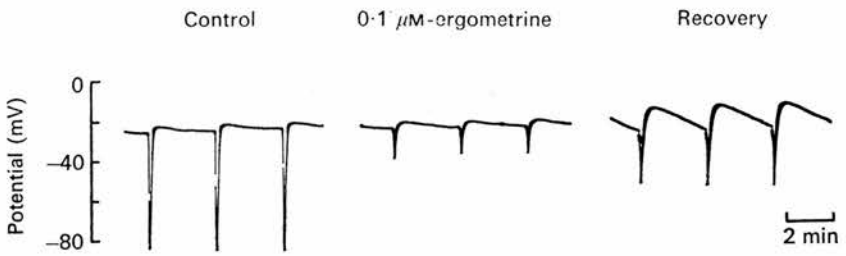


Fig. 10. Effect of ergometrine on the electrical response of an acinar cell to nerve stimulation. Intracellular recordings show biphasic responses to trains of stimuli delivered at intervals of 3 min before, during and after application of ergometrine. These responses are representative and have been extracted from a continuous record of membrane potential. The fourth response illustrated was recorded about 10 min after ergometrine was admitted to the chamber. The seventh was obtained about 30 min after ergometrine was washed off the gland. During both the control and exposure to the inhibitor 9 stimuli at 100 Hz were given to the nerve whereas in the recovery period the train contained 100 stimuli.

Although several compounds have proved useful for inhibiting dopamine responses no specific antagonist is available at present. In the preceding paper (House & Smith, 1978) we examined the effects on secretion of methysergide and ergometrine which block the electrical response of the acinar cells to dopamine (Ginsborg *et al.* 1976). Unlike phentolamine these drugs failed to reduce secretion elicited by dopamine or nerve stimulation. Table 3 shows the effects of these antagonists in other tissues. Evidently their success is mixed and ergometrine, in particular, has agonist activity.

Where intracellular responses to dopamine have been recorded the results suggest the presence of at least two kinds of dopamine receptor. In molluscan neurones the hyperpolarising phase of the biphasic dopamine response is selectively blocked by ergometrine (Ascher, 1972; Berry & Cottrell, 1975). Biphasic responses to nerve stimulation and applied dopamine have been recorded in the cockroach acinar cells and ergometrine seems to be able to selectively suppress the hyperpolarization (Fig. 10). In contrast to phentolamine the recovery to ergometrine was slow and incomplete and occasionally led to an enhancement of the secondary depolarization. Selective reduction of the hyperpolarization seems to be achieved by methysergide also (see Fig. 7, Ginsborg *et al.* 1976).

Mutually opposing responses to dopamine are also found *in vivo*. For example, dopamine infused at high concentrations into cats and dogs produces a rise in blood pressure. This increase masks a transient fall in pressure (Yeh, McNay & Goldberg, 1969), which can be uncovered by selectively inhibiting the rise with $\alpha$-adrenergic antagonists including phentolamine (van Rossum, 1965; McNay & Goldberg, 1966). The fall in pressure has been attributed to activation of specific

dopamine receptors in the renal vasculature (van Rossum, 1966; Yeh *et al.* 1969). This effect can be blocked by ergometrine (Bell *et al.* 1974) and neuroleptic drugs (Goldberg & Yeh, 1971). These compounds probably come closest to being specific dopamine antagonists (e.g. Clement-Cormier, Kebabian, Petzold & Greengard, 1974; Miller, Horn & Iversen, 1974) and the *cis* isomer of flupenthixol is about the most potent of the group. This substance blocks both the electrical and secretory responses to nerve stimulation and dopamine (House & Ginsborg, 1976; C. R. House & J. Breward, unpublished).

Our quantitative study of phentolamine's action has given an affinity constant of about 1 $\mu M^{-1}$ for the catecholamine receptors in the cockroach salivary gland. This value is about 100–1000 times less than is usually found for $\alpha$-receptors (Furchgott, 1972). The electrical response of the acinar cells, however, is probably not mediated by classical adrenergic receptors since selective $\alpha$- and $\beta$-agonists were ineffective at concentrations below 100 $\mu M$ (Ginsborg *et al.* 1976). Thus the hypothesis that dopamine is the transmitter at this neuroglandular junction remains unscathed by the present results and may be used as a basis for future experiments.

We are indebted to Professor B. L. Ginsborg for his comments on the manuscript and to the Science Research Council for financial support. F.B-R. was supported by a James Tindal Scholarship from the Faculty of Veterinary Medicine, University of Edinburgh.

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# THE ACTIONS OF SOME PUTATIVE NEUROTRANSMITTERS ON THE COCKROACH SALIVARY GLAND

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SUMMARY

1. Certain putative transmitters were applied to the innervated cockroach salivary gland and their effects on the resting potential and the neurally evoked secretory potential of the acinar cells were observed.

2. γ-Aminobutyric acid, glutamate, glycine, aspartate and alanine had no significant effect on the resting potential. However, γ-aminobutyric acid and glutamate reduced the neurally evoked secretory potential but only at concentrations above $10^{-3}$ M.

3. Acetylcholine and carbachol appeared to act by modifying transmitter output from the salivary nerves. These substances failed to have any effect on the resting potential.

4. The biogenic amines, adrenaline, dopamine, noradrenaline, 5-hydroxytryptamine and octopamine, produced hyperpolarizing responses, graded according to concentration,

5. It is suggested that dopamine, the most potent of the biogenic amines tested, is the transmitter at this junction.

## INTRODUCTION

Intracellular recordings from acinar cells of the cockroach salivary gland reveal that a hyperpolarizing response can be evoked by electrical stimulation of the salivary nerve (House, 1973, 1975). This response evidently arises from an increase in membrane potassium permeability (Ginsborg, House & Silinsky, 1974), although the participation of other permeability changes has not been excluded. It was noted in previous experiments (House, 1973; House, Ginsborg & Silinsky, 1973) that certain biogenic amines, namely 5-hydroxytryptamine (5-HT), adrenaline, noradrenaline, and dopamine, also cause hyperpolarization. These results are of interest because the presence of catecholamines, but not 5-HT, has been demonstrated in the salivary nerve terminals by microspectrofluorimetry (Bland *et al.* 1973). Furthermore, a radiochemical assay for catecholamines confirmed the presence of dopamine but not adrenaline or noradrenaline (Fry, House & Sharman, 1974).

It was therefore of interest to examine further the effects of these amines on the membrane potential of the acinar cells and also to study the actions of other putative transmitters. The present paper describes the results of these experiments.

## METHODS

Experiments were made at room temperature on the isolated salivary glands of adult cockroaches, *Nauphoeta cinerea* (Oliv.), reared as described previously (House, 1973). Dissected glands were bathed in a solution (pH 6·9) containing 160 mM-NaCl, 1 mM-KCl, 5 mM-$CaCl_2$, 1 mM-$NaHCO_3$ and 1 mM-$NaH_2PO_4$ and mounted in a chamber described by Ginsborg *et al.* (1974).

Membrane potentials were recorded with glass microelectrodes filled with 3 M-KCl by the method of Thomas (1972); the resistance of these electrodes lay in the range 20–40 MΩ. The microelectrode was connected to the input of a wide-band unity gain Bak electrometer and its potential was measured relative to that of an Ag/AgCl pellet fixed in the chamber. Membrane potentials were displayed on a Tektronix 502A oscilloscope and recorded with a Devices M2 recorder. It has been demonstrated in other experiments with procion-filled electrodes (House, 1975) that genuine intracellular recordings are made with the present methods based on previous criteria (House, 1973) for intracellular impalement.

The salivary nerves attached to the gland ducts were drawn into a suction electrode and stimulated by short trains (1–100 at 100 Hz) of pulses (0·5 ms, 10–60 V) from a square pulse stimulator. The interval between successive trains was usually 2–3 min.

The bathing solution was pumped through the chamber (volume 4·5 ml) at about 2 ml. $min^{-1}$ by a Watson Marlow H. R. flow inducer (MHRE 200) except when drug solutions were applied to the preparation at a perfusion rate of about 20 ml. $min^{-1}$. No flow artifacts were observed in the electrical records. The time course of the agonist responses was not affected by the perfusion rate provided it exceeded about 7 ml. $min^{-1}$.

The effects of the following substances were tested on the membrane potential. Acetylcholine (ACh), adrenaline, L-alanine, γ-aminobutyric acid (GABA), L-aspartic acid, carbachol, dopamine, glycine, 5-hydroxytryptamine (5-HT), octopamine, picrotoxin and noradrenaline were supplied by the Sigma Chemical Co., atropine, L-glutamic acid and physostigmine by B.D.H. and (+)-tubocurarine chloride by Koch-Light Lab. Stock solutions of these substances were prepared immediately before use and suitable serial dilutions were made with the physiological saline given above. The pH of all solutions was checked and appropriate adjustments made with 10 N-NaOH to give pH 6·9. During the course of experiments the solutions of the biogenic amines, ACh and carbachol, were frequently remade because of their lability at high dilutions.

## RESULTS

The aim of the investigation was to establish whether or not certain putative neurotransmitters could act at the neuroglandular junctions of the cockroach salivary gland. It was considered that some of these substances might act *directly* on the gland cell membrane and mimic the electrical response evoked by the actual transmitter. Another possibility is that they might exert *indirect* effects at the junction by, for example, altering the amount of transmitter released; there are, of course, other possible modes of indirect action. In order to decide if a particular substance acts directly or indirectly we monitored not only the resting potential but also the ampli-

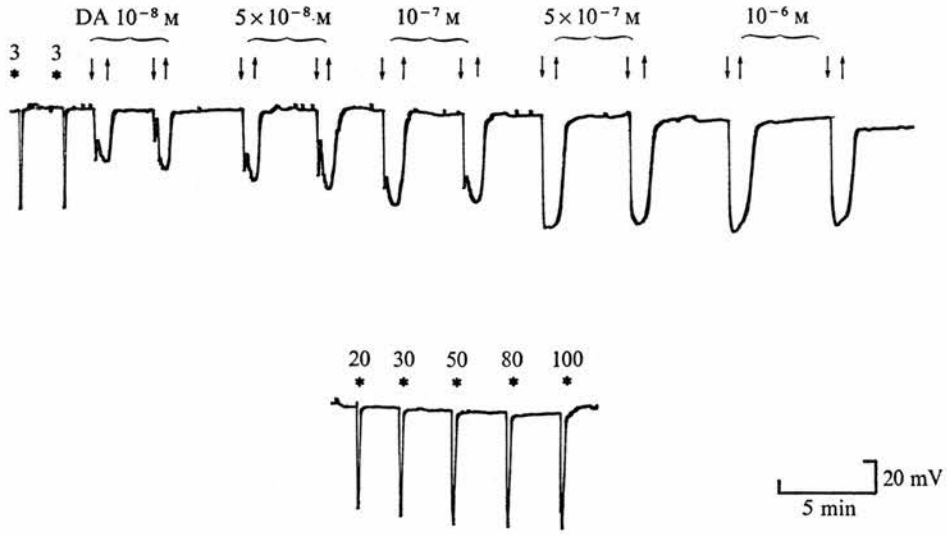


Fig. 1. Intracellular recording showing the effects of dopamine (DA) and nerve stimulation on the resting potential. The duration of application of dopamine is the interval between the arrows. Responses evoked by nerve stimulation are marked by asterisks and the number of stimuli delivered is given above each response. The maximum responses to nerve stimulation (lower trace) were obtained after the dopamine responses (upper trace). The resting potential of this cell was approximately −35 mV.

tude of the neurally evoked response. When this response is compared with one produced by an agonist, such as dopamine, we will refer to the former as the evoked response; otherwise it will be called the secretory potential, as is customary.

### *Biogenic amines*

A number of biogenic amines have been shown to hyperpolarize the acinar cells (House, 1973; House *et al.* 1973), and it was decided to investigate their dose-response relationships more fully than previous work had allowed. Each dose-response curve was obtained from a cell in a different preparation.

The results of a typical experiment with dopamine are shown in Fig. 1. The evoked responses (marked by asterisks) attained a maximum amplitude of about 75 mV in this cell (lower trace); the time course of such responses can be seen more clearly in other recordings (Figs. 4A and 5D). Generally, the maximum responses of cells to nerve stimulation were about 70 mV and occurred when there were 30 to 100 stimuli in the train. A gradation of response size with the number of stimuli was found between 1 and 30. The upper trace (Fig. 1) shows responses to dopamine applied at different concentrations in the bathing fluid. The time course of the dopamine responses is longer than that of the evoked responses, presumably because of its relatively slow application. It has already been demonstrated that the duration of the evoked response can be lengthened to match such agonist responses by maintaining nerve stimulation for a suitable period (Bland *et al.* 1973). The amplitude of the dopamine response depends on its concentration and the maximum attained is the same as that for the evoked response.

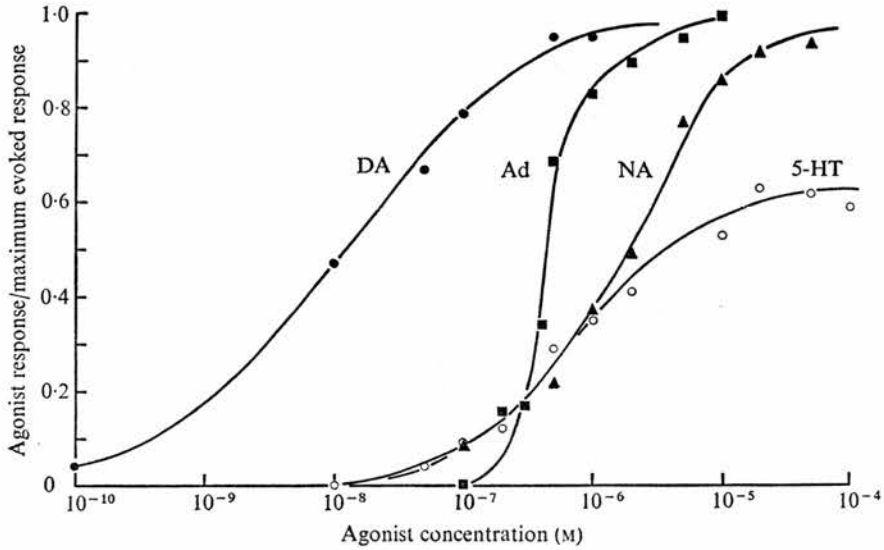


Fig. 2. Typical log dose-response curves for certain biogenic amines. Each agonist response has been divided by the corresponding maximum evoked response for each cell. These maxima were 76, 65, 58 and 68 mV for dopamine (DA), noradrenaline (NA), adrenaline (Ad) and 5-hydroxytryptamine (5-HT) curves, respectively.

Two cells of the 11 examined had a high sensitivity to dopamine; the dose-response curve for one of these is shown in Fig. 2. In both cases the threshold concentration for electrical responses was about $10^{-10}$ M whereas the usual threshold was about $10^{-9}$ M. One of the cells studied had a threshold of $5 \times 10^{-8}$ M; we are at a loss to account for its low sensitivity since the maximum dopamine response attained was similar to that of the more sensitive cells.

Noradrenaline, adrenaline and 5-HT are also agonists as judged by their ability to hyperpolarize acinar cells. Dose-response curves for these substances and also dopamine are shown in Fig. 2, each agonist being studied in a different cell. These particular examples have been selected to emphasize the differences between the curves. To facilitate comparison of results obtained on different cells the agonist responses from each cell have been normalized by dividing their amplitudes by that of the corresponding maximum evoked responses. The values of these maxima are given in the figure legend. It was not possible to obtain curves for all agonists in the same cell as the period of stable recording was invariably too short. However, the dose-response curves illustrated in Fig. 2 reflect the general trends observed in 11 cells with dopamine, 9 with noradrenaline, 5 with adrenaline and 6 with 5-HT. All the agonists except 5-HT were able to generate a maximum response equal to the maximum evoked response. Table 1 gives the mean values of the agonist concentrations required to yield a half-maximum response and also the slopes of the linear portions of the log dose-response curves. Dopamine is clearly the most potent agonist, noradrenaline and adrenaline are almost equipotent, and 5-HT is apparently only a partial agonist since it failed to produce a maximum equal to that of the evoked response.

The average slope for adrenaline is steeper than for dopamine and noradrenaline,

Table 1. *Parameters of dose-response curves for different agonists*

| Agonist | Mean ± S.E. Concentration (M) required to give half-maximum agonist response | Mean ± S.E. Slope of log dose-response curve (mv/decade) |
|---|---|---|
| Dopamine (11) | $(4{\cdot}2 \pm 1{\cdot}6) \times 10^{-8}$ | $38 \pm 3{\cdot}4$ |
| Noradrenaline (9) | $(1{\cdot}3 \pm 0{\cdot}40) \times 10^{-6}$ | $41 \pm 3{\cdot}9$ |
| Adrenaline (5) | $(1{\cdot}5 \pm 0{\cdot}50) \times 10^{-6}$ | $69 \pm 13$ |
| 5-HT (6) | $(3{\cdot}7 \pm 0{\cdot}87) \times 10^{-7}$ | $25 \pm 4{\cdot}7$ |

The number of experiments is given in parentheses after each agonist.

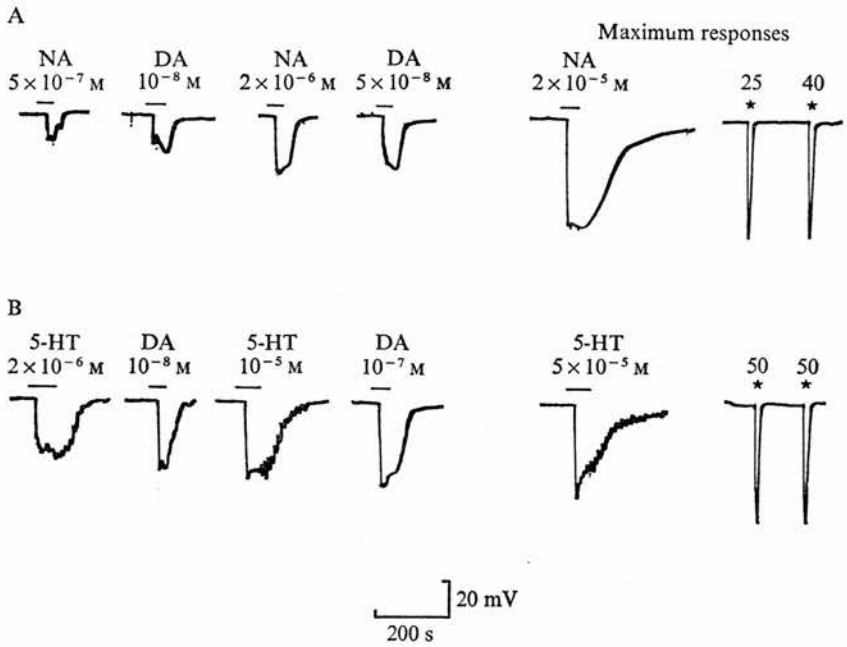


Fig. 3. Comparison of responses to different biogenic amines recorded in the same cells. The duration of agonist application is indicated by the horizontal bars. (A) Responses to noradrenaline (NA) and dopamine (DA). Maximum responses to NA and nervous stimulation (number of stimuli above asterisks) are also shown. (B) Similar results for 5-HT and DA in a different cell. The neurally evoked maximum responses were obtained after the agonist responses and were equal to those (not shown) recorded at the beginning of the experiment. The resting potential of the cell in A was −30 mV and in B −34 mV.

and this suggests that the receptor for adrenaline is different from the other catecholamine receptors.

The conclusion that dopamine is more potent than noradrenaline and 5-HT is supported by additional experiments where the effects of these agonists were compared in the same cell. For example, Fig. 3 shows recordings made in two different cells where equivalent responses to noradrenaline and dopamine were obtained in the first cell (3A) and to 5-HT and dopamine in the second (3B). In 3A the dose ratios required for equal responses were about 50 NA:1 DA whereas in 3B they were

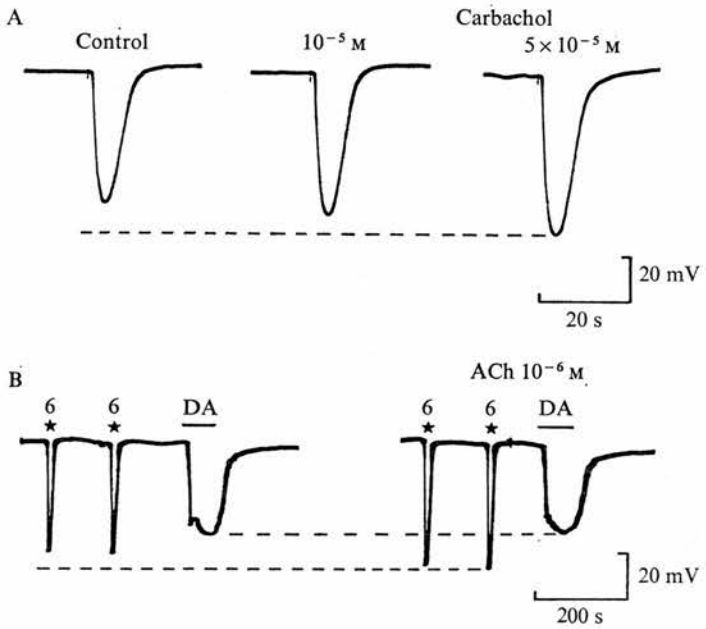


Fig. 4. Effects of carbachol and ACh on the neurally evoked secretory potentials of two cells. (A) Graded increase in the evoked responses in the presence of carbachol at two different concentrations. The resting potential was −40 mV. (B) Similar effect of ACh on the evoked response whereas the response to dopamine (DA) at $5 \times 10^{-8}$ M was unaffected. Physostigmine ($1{\cdot}3 \times 10^{-5}$ M) was present before and during ACh application. The resting potential of this cell was −28 mV.

about 150 5-HT: 1 DA. A further point of interest is that noradrenaline, like dopamine (Fig. 1), is able to elicit responses equal to the maximum evoked responses whereas 5-HT cannot. In fact, the maximum 5-HT response was not maintained in this cell although the duration of application was similar to that for noradrenaline. The reason for this is not clear but it might be due to a greater desensitization to 5-HT than to the other agonists studied.

Another biogenic amine of possible significance is octopamine, which has been found in the nervous systems of mammals (Molinoff & Axelrod, 1969), crustacea (Molinoff & Axelrod, 1972), molluscs (Walker, Ramage & Woodruff, 1972) and insects (Robertson & Steele, 1973). In contrast to the amines examined above, octopamine is a poor agonist, producing only a small hyperpolarization at a concentration of $10^{-5}$ M. Even at concentrations of $10^{-4}$–$10^{-3}$ M it gave responses less than 25% of the maximum evoked responses (4 experiments). Applied at $10^{-7}$ M, octopamine increased the secretory potentials by about 30%, and at $10^{-5}$ M the increase was followed by a pronounced decline during prolonged exposure (15 min). Octopamine's ability to alter the size of the secretory potential has not been investigated further.

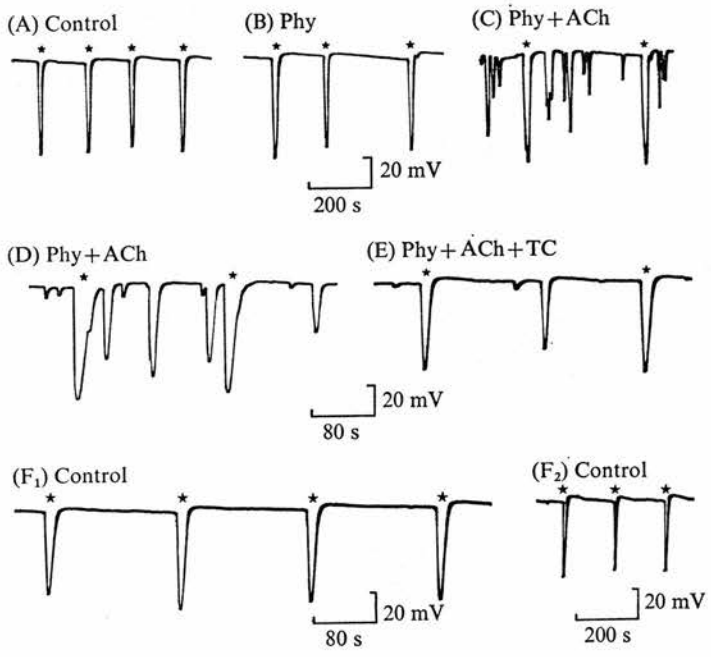


Fig. 5. Actions of acetylcholine (ACh) and (+)-tubocurarine (TC) in the presence of physostigmine (Phy) on the secretory potentials of a gland cell. The secretory potentials evoked by a single stimulus are marked by asterisks. Unmarked changes in potential are spontaneous. (A–F) Extracts from a continuous record. B shows that Phy at $1{\cdot}3 \times 10^{-5}$ M, applied 12 min previously at the end of A, produced a small increase in the evoked response. C and D illustrate that ACh produced a further increase in the evoked response accompanied by spontaneous potentials, these traces being obtained 8 and 33 min after the end of B. E shows that addition of TC at $1{\cdot}3 \times 10^{-5}$ M markedly reduced the effects produced by ACh+Phy, this trace being obtained 50 min after the end of D. F Secretory potentials recorded 5–15 min ($F_1$) and 30–35 min ($F_2$) after washout with physiological saline.

### *Acetylcholine and carbachol*

Neither ACh nor carbachol mimicked nerve stimulation even when applied at concentrations as high as $10^{-3}$ M. Carbachol, however, increased the secretory potential when its concentration was equal to or above $10^{-5}$ M (Fig. 4A). Above $5 \times 10^{-5}$ M it caused spontaneous hyperpolarizations with a similar time course to the secretory potentials evoked by small numbers of stimuli. In the presence of the anticholinesterase, physostigmine, ACh also increased the secretory potential (Fig. 4B) and at higher concentrations it also elicited spontaneous potentials (Fig. 5C, D) similar to those caused by carbachol. The recordings in Fig. 4B demonstrate that ACh can increase the secretory potential without changing the sensitivity of the cell to dopamine, since there was no increment in the submaximal dopamine responses. Thus, it is plausible to suppose that ACh acts on the nerve terminals and enhances transmitter release. This proposal seems compatible with the occurrence of spontaneous potentials in the presence of ACh (Fig. 5C, D) which normally are rarely observed in this gland (House, 1973). Nevertheless, other hypotheses are not excluded by these results, and the effects of ACh and carbachol require further study.

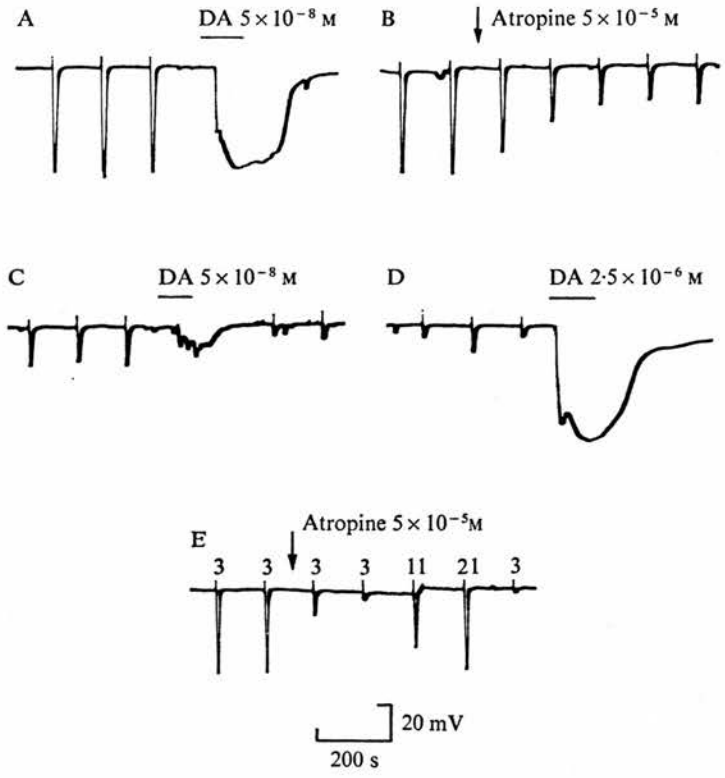


Fig. 6. The blocking action of atropine on the dopamine and neurally evoked response of two gland cells. The records A to D are consecutive, the application of dopamine is indicated by the bars and atropine is present from the arrow in trace B, through C and D. E shows that the atropine blockade of the neurally evoked response can be surmounted by increasing the number of nerve stimuli in the volley; the number of stimuli is indicated above each response. The resting potential in each case was −24 mV.

As ACh can influence transmission at this junction we decided to investigate the effects of cholinergic blocking agents. Fig. 5 displays the results of an experiment with (+)-tubocurarine. In order to obtain an effect from ACh the tissue was pretreated with physostigmine which itself produced a small increment in the amplitude of the secretory potentials (marked by asterisks) (cf. 5A, B). When ACh was applied at $10^{-4}$ M it caused a further increase in the secretory potential and also the emergence of spontaneous potentials (5C, D). Prolonged exposure (20–30 min) to (+)-tubocurarine at $1{\cdot}3 \times 10^{-5}$ M abolished the increment in the secretory potentials produced by ACh and also reduced the frequency and size of the spontaneous potentials (5E). The secretory potentials recorded 5–15 min after washout of these drugs with physiological saline (5 $F_1$) were similar to those observed initially (5A); moreover, the preparation was not affected adversely by these substances as judged by the responses recorded 30–35 min after washout (5 $F_2$). In the absence of physostigmine, ACh failed to generate the effects shown in Figs. 4 and 5 even at concentrations as high as $10^{-3}$ M. These experiments suggest that there are cholinergic receptors, possibly nicotinic, in the salivary nerve terminals. The release of transmitter, however, is not obligatorily dependent on the activation of these receptors since two further experi-

ments also demonstrated that (+)-tubocurarine did not abolish the evoked response. It was therefore of interest to examine the effects of a muscarinic blocker. Atropine was found to reduce the size of the evoked response (Fig. 6A, B). However, the response to dopamine was also diminished (6A, C), but could be restored to its original magnitude by a 50-fold increase in the dopamine concentration. This suggests that atropine acts postjunctionally in this preparation probably in a competitive manner, its affinity constant for the dopamine receptors being about $10^6$ $M^{-1}$. This is roughly, 100 to 1000 times less than values for muscarinic receptors in mammalian preparations (see Barlow, 1964). Additional evidence suggesting that the action of atropine on the evoked response is also postjunctional was obtained in three experiments where it was found that atropine block could be surmounted by increasing the number of nerve stimuli (Fig. 6E). The possibility that atropine acts as a local anaesthetic at the concentrations (*ca.* $5 \times 10^{-5}$ M) used in our experiments has not been excluded, although it seems unlikely in view of its failure at $10^{-4}$ M to reduce the size of the action potential of mammalian C fibres (Armett & Ritchie, 1961).

Thus, atropine can substantially reduce the evoked response probably by interacting with the gland cell receptors for the transmitter. In contrast, it is likely that tubocurarine blocks cholinergic receptors on salivary nerve terminals.

### *Amino acids*

The view that certain amino acids are neurotransmitters is supported by evidence reviewed recently by Gerschenfeld (1973) for invertebrates and Krnjević (1974) for vertebrates. In particular, the case for GABA as the inhibitory transmitter at the neuromuscular junctions in certain crustaceans is compelling (see e.g. Otzuka *et al.* 1966). GABA is also likely to have a similar role in insects. It seemed worthwhile, therefore, to test whether or not this substance is responsible for the secretory potential. In 10 experiments it was applied at concentrations in the range $10^{-8}$–$10^{-2}$ M. No effects on the resting potential were observed, but at concentrations above or equal to $10^{-4}$ M, GABA produced a small reduction (10%) in the secretory potential. In four experiments it was shown that picrotoxin, known to be an antagonist of GABA (Robbins & Van der Kloot, 1958), did not alter the resting or secretory potential when applied at concentrations of $10^{-4}$ and $10^{-3}$ M for periods of up to 20 min. Clearly GABA does not mimic the transmitter nor does it exert impressive effects on the secretory potential.

Another substance worth studying is L-glutamic acid, whose role as an excitatory neuromuscular transmitter in crustacea and insects is supported by several lines of evidence (see Gerschenfeld, 1973). In six experiments it was applied in concentrations in the range $10^{-8}$–$10^{-2}$ M. We observed, as for GABA, a small reduction (10%) in the size of the evoked response but only at the very high concentration of $10^{-2}$ M. No changes in the resting potential were observed.

The effects of other putative neurotransmitters, namely glycine, L-alanine and L-aspartic acid, were investigated at concentrations in the same range as above for GABA and glutamic acid. Each substance was tested on 3 preparations and none produced effects on resting or secretory potentials.

## DISCUSSION

Of the eleven putative transmitters tested, only the catecholamines, adrenaline, noradrenaline and dopamine, are able to mimic closely the effects of the actual transmitter. Dopamine is clearly the most potent of these agonists. It is difficult to separate adrenaline and noradrenaline on the basis of potency but the slope of the log dose-response curve for adrenaline is significantly different from those for noradrenaline and dopamine. This suggests that the gland cell receptors are not classically adrenergic. Further evidence supporting this conclusion is the failure of certain $\alpha$- and $\beta$-agonists to produce electrical responses (House *et al.* 1973). Our tentative conclusion is that the dopamine receptors are identical to those for the actual transmitter. At present it is impossible to differentiate between the dopamine receptors and those for noradrenaline. However, our present results taken together with the previous evidence (Bland *et al.* 1973; House *et al.* 1973; Fry *et al.* 1974) indicate that dopamine, rather than noradrenaline, is likely to be the transmitter at this junction. The only other biogenic amine tested, apart from 5-HT, was octopamine which turned out to be a very weak agonist; like both adrenaline and noradrenaline it also has not been detected in this tissue (Fry, House and Sharman, unpublished).

It is worth noting here that the related amine, *N*-acetyldopamine, is present in many species of insect (see review by Murdock, 1971), including the cockroach (Mills *et al.* 1967) and is known to be the tanning agent in the blowfly (Karlson & Sekeris, 1962). However, this substance fails to change the resting or secretory potential in this gland (unpublished observations) and, therefore, it probably plays no part in neuroglandular transmission in this insect.

Whereas it is not yet possible to distinguish the effects of dopamine from those of noradrenaline, it is quite clear that the 5-HT receptors are different from those for dopamine (House *et al.* 1973). Our experiments showed that 5-HT could not generate a maximum electrical response as large as the maximum evoked response. It seems highly unlikely that 5-HT is the transmitter in this tissue because its presence has not been detected in the nerve terminals (Bland *et al.* 1973) and, moreover, the 5-HT receptors in the gland cells are different from those for the actual transmitter (House *et al.* 1973). There is evidence, however, that it is the neurohormone for the uninnervated salivary gland of the blowfly (Berridge & Patel, 1968; Berridge, 1972).

The evidence against a direct transmitter rôle for ACh, GABA, glutamate, glycine, alanine and aspartate is unequivocal since none of them produces an electrical response. In the case of ACh it could be argued that it is involved perhaps in augmenting the release of the transmitter. Certainly it causes spontaneous potentials and also enhances evoked responses if an anticholinesterase is present. Such a proposed interaction could be thought of as *presynaptic excitation* whereby ACh released from one type of nerve terminal potentiates the release of the actual transmitter from another. Hypotheses of cholinergic links in adrenergic transmission have taken several forms since that first proposed by Burn & Rand (1959). There is at present no compelling evidence for any of these proposals, including the one mentioned above, although there is at least some anatomical evidence for axo-axonal synapses between adrenergic and cholinergic nerve terminals (Ehinger, Falck & Sporrong, 1970). However, our knowledge of the innervation of this salivary gland is too fragmentary

to allow us to make an attractive analogy. Clearly more experimental evidence is required before a sensible conclusion can be drawn about the rôle of ACh in this tissue.

Although the experiments with GABA and glutamate show that these substances can influence the size of the secretory potential, the effects occur only at very high concentrations and, therefore, appear at present to be of pharmacological interest rather than of physiological significance.

Our main conclusion is that the transmitter in the cockroach salivary gland is probably dopamine. The evidence for this is circumstantial just as it is for a similar rôle for dopamine in the salivary glands of the locust (Klemm, 1972) and ticks (Kaufmann & Phillips, 1973; Megaw & Robertson, 1974).

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